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(21) International Application Number: PCT/US99/26334 (22) International Filing Date: 8 November 1999 (08.11.99) (30) Priority Data: 09/187,387 6 November 1998 (06.11.98) US (71) Applicants (for all designated States except US): BIOGEN, INC. [US/US]; Fourteen Cambridge Center, Cambridge, MA 02142 (US). ONTOGENY, INC. [US/US]; 45 Moulton Street, Cambridge, MA 02138 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): GALDES, Alphonse [US/US]; 1 Douglas Road, Lexington, MA 02173 (US). MAHANTHAPPA, Nagesh [US/US]; 240 Norfolk Street, Cambridge, MA 02139 (US). (74) Agents: VINCENT, Matthew, P. et al.; Foley, Hoag & Eliot, LLP, One Post Office Square, Boston, MA 02109 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: METHODS AND COMPOSITIONS FOR TREATING OR PREVENTING PERIPHERAL NEUROPATHIES (57) Abstract The present application is directed to the discovery that hedgehog gene products are able to protect peripheral nerve cells under conditions which otherwise result in peripheral neuropathy. Certain aspects of the invention are directed to preparations of hedgehog polypeptides, or other molecules which regulate <i>patched</i> or <i>smoothened</i> signalling, and their uses as protective agents against both acquired and hereditary neuropathies. As used herein, "peripheral neuropathy" refers to a disorder affecting a segment of the peripheral nervous system. For instance, the method of the present invention can be used as part of a treatment program in the management of neuropathies associated with systemic disease, e.g., viral infections, diabetes, inflammation; as well as genetically acquired (hereditary) neuropathies, e.g., Charcot-Marie-Tooth disease; and neuropathies caused by a toxic agent, e.g., a chemotherapeutic agent such as vincristine.		

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*Methods and Compositions for Treating
or Preventing Peripheral Neuropathies*

Background of the Invention

5 Conditions that affect components of a motor unit (motor neuron cells of the spinal cord, nerve, neuromuscular junction, and muscle fibers), sensory and autonomic nerves or their supportive structures are included in the broad category of "neuromuscular disorders", and include peripheral neuropathies.

10 Motor nerves are responsible for voluntary movement. Their cell bodies lie within the spinal cord, and their processes transmit signals outward to specialized motor receptors on the skeletal muscles. Sensory nerves allow the sensation of pain, vibrations or touch, and sense where limbs are positioned in space. Their cell bodies are grouped in specialized structures called sensory "ganglia" next to the spinal cord. And they transmit signals from sensory receptors in the skin and other organs inward to the central nervous
15 system (CNS). Autonomic nerves control involuntary functions like breathing, heartbeat, blood pressure, digestion and sexual function. Their cell bodies, clustered in autonomic ganglia, are spread throughout the body.

20 Neuropathy is a generic term used to describe diseases of the peripheral nervous system. There are about 200 known different causes of peripheral neuropathies. Although most neuropathies affect all three types of nerve fibers, to varying degrees, some diseases involve only one or two, and are thus said to be purely or predominantly motor, sensory, or autonomic neuropathies.

25 For instance, Guillain-Barré syndrome is an acute illness involving the peripheral nervous system that usually occurs two to three weeks after a flu-like disease or other infections. It is mostly a motor neuropathy, meaning that its symptoms are largely related to the involvement of the motor nerves. Despite the primarily motor nature of the disease, the earliest symptoms may be numbness and tingling felt in the lower extremities followed shortly by weakness of the distal muscles of the lower extremities. The common early symptoms reported by patients are those of tripping on the toes that
30 later results in a footdrop. The weakness usually ascends to involve the entire lower extremities and later the upper extremities. The danger occurs when the weakness involves the muscles of respiration.

 The diagnosis of Guillain-Barré syndrome is suggested when the patient presents with a history of ascending weakness and a physical examination consistent with a

primarily motor neuropathy. The diagnosis is confirmed with the performance of a spinal tap, which usually shows elevation of the protein level in the spinal fluid without an increase in the number of white cells and by an electromyogram. All other conditions resembling Guillain-Barré syndrome must also be excluded.

- 5 Although Guillain-Barré syndrome is usually a self-limiting illness, intensive therapeutic intervention is often needed.

CIDP or chronic inflammatory demyelinating polyneuropathy is an immune-mediated neuropathy that affects the peripheral motor and sensory nerves. The symptoms are of a slowly progressive numbness and tingling that usually starts in the
10 feet, but later spreads to the legs and hands. The patients also complain of some weakness, again usually starting in the lower extremities, but soon involving the upper extremities. With further involvement of the sensory system, other modalities of sensations, such as balance, are affected and the patients complain of inability to walk or maintain balance in the dark.

- 15 The diagnosis of CIDP is suspected with a history of progressive sensorimotor neuropathy. Physical examination consistent with distal sensory loss in the upper and lower extremities, in conjunction with motor weakness that can be more proximal than distal supports the clinical diagnosis. The spinal tap usually shows a significant rise in the protein level of the spinal fluid. Electromyography with nerve conduction studies
20 also supports the diagnosis. Usually the main picture is that of slowing of the conduction velocities of the peripheral nerves. The final diagnostic step would be the performance of a nerve biopsy. Finding of inflammation on the nerve biopsy, although rare, definitely confirms the diagnosis. However, the absence of inflammation does not entirely rule it out. Findings of predominant demyelination on the nerve biopsy can be used in
25 conjunction with the other studies and the clinical presentation to suggest a diagnosis of CIDP. Once the diagnosis is secured, treatment with immunosuppressive medications can be initiated. The first line of treatment remains high-dose steroids that are initiated orally every day and then slowly tapered over time depending on the patient's improved symptomatology. Steroid failure or intolerance to steroids necessitates the use of other
30 immunosuppressing agents. However, better therapeutic intervention for CIDP is still a desired objective of the present invention.

- Peripheral neuropathy is one of the many complications of long-standing diabetes. Usually neuropathy occurs about 8 to 10 years after the onset of diabetes. However, it is not uncommon to see patients presenting with neuropathic symptoms that
35 have their diabetes diagnosed at that time or patients with 20 or more years of diabetes with little or no evidence of neuropathy. The symptoms of diabetic neuropathy consist of

a slow and insidious numbness and tingling of the lower extremities that can progress to become a painful neuropathy. The pain is usually described as a burning sensation in the feet. Occasionally, the pain is described as a sensation of sharp, electric jolts traveling down the lower extremities. As it worsens, the pain acquires a deep bony nature. It tends to be worse at night commonly preventing or awakening the patients from sleep. As the neuropathy worsens, it affects the upper extremities and may involve the motor nerves with the complaint of weakness in the distal muscles of the legs and arms. The neuropathy of diabetes can also involve the autonomic nervous system causing problems with sweating, blood pressure, and sexual function.

Diabetic neuropathy is suspected when the patient's history and physical examination are compatible with the clinical picture in a setting of diabetes. In the absence of the history of diabetes, diagnostic tests to rule out diabetes is required. The workup is completed by the performance of an electromyogram with nerve conduction studies to quantitate the extent of involvement of the peripheral nervous system.

Diabetic neuropathy, unfortunately, has no effective treatment at this point in the art. Adequate control of the patient's blood sugar, however, has been shown to slow the progression of the symptoms. Symptomatic treatment with various medications that suppress neuropathic pain, including Elavil, Tegretol and more recently Ultram, have been successful. Thus, a more effective treatment for diabetic neuropathy is an objective of the present invention.

Other common causes of neuropathy such include alcoholism or medication induced neuropathies, as well as inherited forms of such disorders.

Summary of the Invention

One aspect of the present application relates to a method for treating or alleviating all or a portion of the symptoms attendant neuromuscular disorders, and in particular, in the treatment of peripheral neuropathies. Briefly, the subject method comprises contacting the afflicted tissue with a hedgehog therapeutic or ptc therapeutic (defined *infra*) in an amount effective to alter the growth state of the treated cells, e.g., relative to the absence of administration of the hedgehog therapeutic or ptc therapeutic.

Wherein the subject method is carried out using a *hedgehog* therapeutic, the *hedgehog* therapeutic preferably a polypeptide including a *hedgehog* portion comprising at least a bioactive extracellular portion of a *hedgehog* protein, e.g., the *hedgehog* portion includes at least 50, 100 or 150 (contiguous) amino acid residues of an N-terminal half of a *hedgehog* protein. In preferred embodiments, the *hedgehog* portion

includes at least a portion of the *hedgehog* protein corresponding to a 19kd fragment of the extracellular domain of a *hedgehog* protein.

5 In preferred embodiments, the *hedgehog* portion has an amino acid sequence at least 60, 75, 85, or 95 percent identical with a hedgehog protein of any of SEQ ID Nos. 10-18 or 20, though sequences identical to those sequence listing entries are also contemplated as useful in the present method. The *hedgehog* portion can be encoded by a nucleic acid which hybridizes under stringent conditions to a nucleic acid sequence of any of SEQ ID Nos. 1-9 or 19, e.g., the *hedgehog* portion can be encoded by a vertebrate *hedgehog* gene, especially a human *hedgehog* gene.

10 In other embodiments, the subject method can be carried out by administering a gene activation construct, wherein the gene activation construct is deigned to recombine with a genomic *hedgehog* gene of the patient to provide a heterologous transcriptional regulatory sequence operatively linked to a coding sequence of the *hedgehog* gene.

15 In still other embodiments, the subject method can be practiced with the administration of a gene therapy construct encoding a *hedgehog* polypeptide. For instance, the gene therapy construct can be provided in a composition selected from a group consisting of a recombinant viral particle, a liposome, and a poly-cationic nucleic acid binding agent,

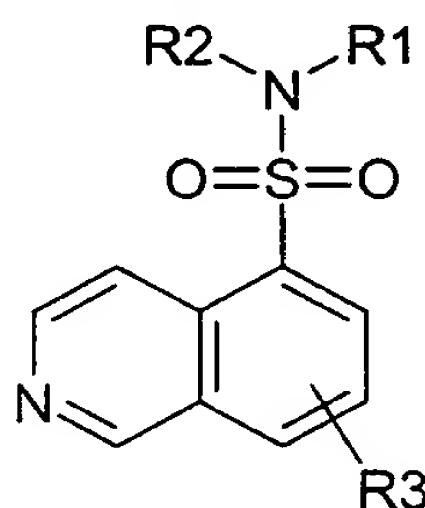
20 In yet other embodiments, the subject method can be carried out using a *ptc* therapeutic. An exemplary *ptc* therapeutic is a small organic molecule which binds to a *patched* protein and derepresses *patched*-mediated inhibition of mitosis, e.g., a molecule which binds to *patched* and mimics *hedgehog*-mediated *patched* signal transduction, which binds to *patched* and regulates *patched*-dependent gene expression. For instance, the binding of the *ptc* therapeutic to *patched* may result in upregulation of *patched* and/or *gli* expression.

25 In a more generic sense, the *ptc* therapeutic can be a small organic molecule which interacts with MK cells to induce *hedgehog*-mediated *patched* signal transduction, such as by altering the localization, protein-protein binding and/or enzymatic activity of an intracellular protein involved in a *patched* signal pathway. For instance, the *ptc* therapeutic may alter the level of expression of a *hedgehog* protein, a *patched* protein or a protein involved in the intracellular signal transduction pathway of *patched*.

30 In certain embodiments, the *ptc* therapeutic is an antisense construct which inhibits the expression of a protein which is involved in the signal transduction pathway of *patched* and the expression of which antagonizes *hedgehog*-mediated signals. The

antisense construct is preferably an oligonucleotide of about 20-30 nucleotides in length and having a GC content of at least 50 percent.

In other embodiments, the *ptc* therapeutic is an inhibitor of protein kinase A (PKA), such as a 5-isoquinolinesulfonamide. The PKA inhibitor can be a cyclic AMP analog. Exemplary PKA inhibitors include N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine, KT5720, 8-bromo-cAMP, dibutyryl-cAMP and PKA Heat Stable Inhibitor isoform α . Another exemplary PKA inhibitor is represented in the general formula:



10 wherein,

R_1 and R_2 each can independently represent hydrogen, and as valence and stability permit a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-R_8$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$ -lower alkyl, $-(CH_2)_m-O$ -lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$, $-(CH_2)_m-SH$, $-(CH_2)_m$ -S-lower alkyl, $-(CH_2)_m-S$ -lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$, or

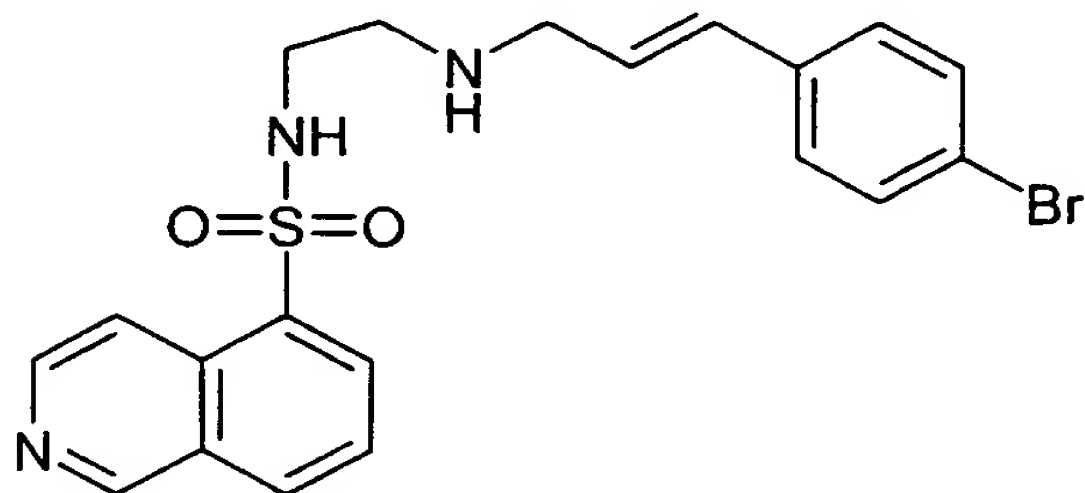
R_1 and R_2 taken together with N form a heterocycle (substituted or unsubstituted);

20 R_3 is absent or represents one or more substitutions to the isoquinoline ring such as a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-R_8$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$ -lower alkyl, $-(CH_2)_m$ -O-lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$ -lower alkyl, $-(CH_2)_m-S$ -lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$;

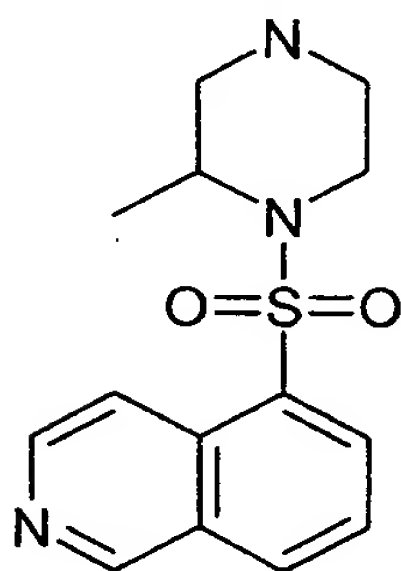
R_8 represents a substituted or unsubstituted aryl, aralkyl, cycloalkyl, cycloalkenyl, or heterocycle; and

n and m are independently for each occurrence zero or an integer in the range of 1 to 6.

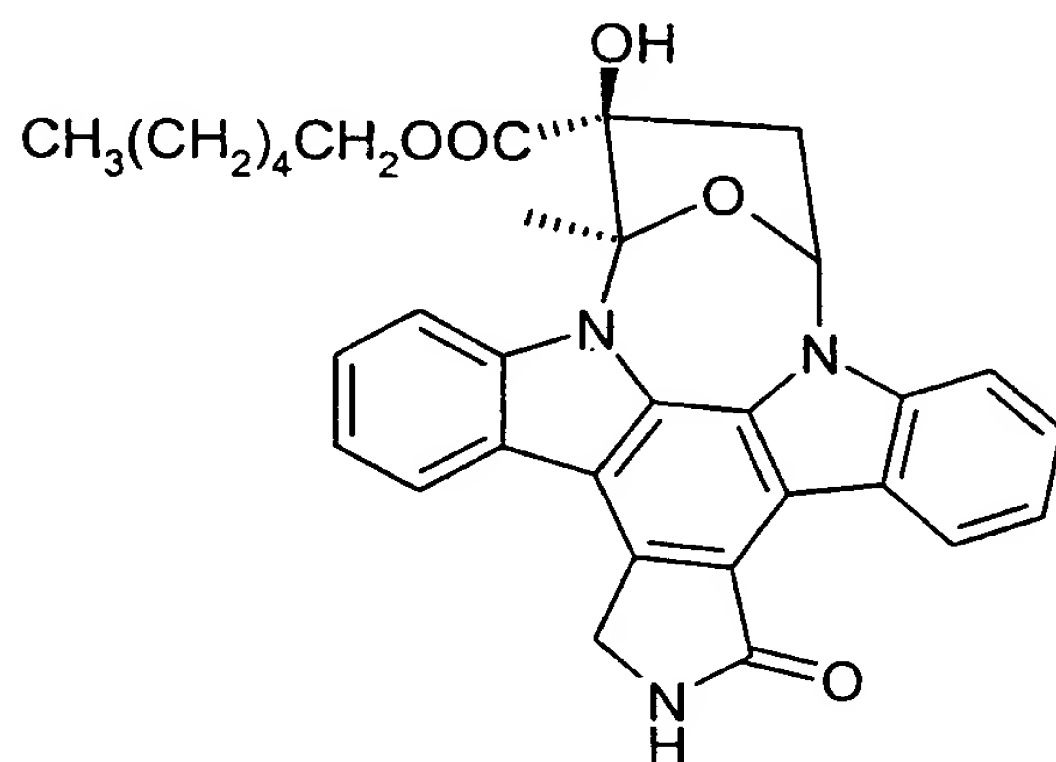
In a preferred embodiment, the PKA inhibitor is N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H-89; Calbiochem Cat. No. 371963), e.g., having the formula:



In another embodiment, the PKA inhibitor is 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7; Calbiochem Cat. No. 371955), e.g., having the formula:



In still other embodiments, the PKA inhibitor is KT5720 (Calbiochem Cat. No. 420315), having the structure



The *hedgehog* pathway can be agonized by antagonizing the cAMP pathway, e.g., by using an agonist of cAMP phosphodiesterase, or by using an antagonist of

adenylate cyclase, cAMP or protein kinase A (PKA). Compounds which may reduce the levels or activity of cAMP include prostaglandylinositol cyclic phosphate (cyclic PIP), endothelins (ET)-1 and -3, norepinephrine, K252a, dideoxyadenosine, dynorphins, melatonin, pertussis toxin, staurosporine, G_i agonists, MDL 12330A, SQ 22536, GDPssS and clonidine, beta-blockers, and ligands of G-protein coupled receptors. Additional compounds are disclosed in U.S. Patent Nos. 5,891,875, 5,260,210, and 5,795,756.

Exemplary peptidyl inhibitors of PKA activity include the PKA Heat Stable Inhibitor (isoform α ; see, for example, Calbiochem Cat. No. 539488, and Wen et al. (1995) *J Biol Chem* 270:2041).

In certain embodiments, a compound which is an agonist or antagonist of PKA is chosen to be selective for PKA over other protein kinases, such as PKC, e.g., the compound modulates the activity of PKA at least an order of magnitude more strongly than it modulates the activity of another protein kinase, preferably at least two orders of magnitude more strongly, even more preferably at least three orders of magnitude more strongly. Thus, for example, a preferred inhibitor of PKA may inhibit PKA activity with a K_i at least an order of magnitude lower than its K_i for inhibition of PKC, preferably at least two orders of magnitude lower, even more preferably at least three orders of magnitude lower. In certain embodiments, a *ptc* therapeutic inhibits PKC with a K_i greater than 10 nM, greater than 100 nM, preferably greater than 1 μ M.

Brief Description of the Figures

Figure 1. Variation of the weight of animals during the study in treated or control mice: control SHH=animals treated with 500 ug/kg SHH, without cisplatin; veh=vehicle group treated with cisplatin 2 mg/kg/day during 14 days; SHH500=animals treated with 500 ug/kg SHH and cisplatin; SHH50=animals treated with 50 ug/kg SHH and cisplatin. The compounds were administered 3 times per week subcutaneously. The weights are expressed in grams, as means \pm SEM. Post-hoc comparison to vehicle group was performed with Fisher test; *:significantly different at $p<0.05$; **:significantly different at $p<0.01$; ***;significantly different at $p<0.001$.

Figure 2. Number of animals present throughout the study in treated or control mice. The number of animals in each group was compared by repeated Anova test and was not found to be significantly different between groups.

5 Figure 3. Time course of sensory nerve conduction velocity (SNCV) measured in treated or control mice. Results are expressed in m/sec, as means \pm SEM. Post-hoc comparison to vehicle group was performed with Fisher test; *:significantly different at $p<0.05$; **:significantly different at $p<0.01$; ***:significantly different at $p<0.001$.

10 Figure 4. Tail flick latency measured in treated or control mice. Results are expressed in sec, as means \pm SEM. Post-hoc comparison to vehicle group was performed with Fisher test; *:significantly different at $p<0.05$; **:significantly different at $p<0.01$; ***:significantly different at $p<0.001$.

Figure 5. Latency to lick the paw measured in treated or control mice. Results are expressed in sec as means \pm SEM. Post-hoc comparison to vehicle group was performed with Fisher test.

15 Figure 6. Latency before first jump measured in treated or control mice. Results are expressed in sec, as means \pm SEM. Post-hoc comparison to vehicle group was performed with Fisher test; *:significantly different at $p<0.05$.

20 Figure 7. Latency before adjusted jump measured in treated or control mice. Results are expressed in sec, as means \pm SEM. Post-hoc comparison to vehicle group was performed with Fisher test.

Figure 8. Ability to stay on rotarod measured in treated or control mice.

25 Figure 9. Duration of the walk on a rod needed to reach the platform, measured in treated or control mice. Results are expressed in sec, as means \pm SEM. Post-hoc comparison to vehicle group was performed with Fisher test; *:significantly different at $p<0.05$; **:significantly different at $p<0.01$; ***: significantly different at $p<0.001$.

Figures 10A and 10B. Ability to hold a weight with four limbs (10a) or only forelimbs (10b) measured in treated or control mice. Results are expressed in sec, as means \pm SEM. Post-hoc comparison to vehicle group was performed with Fisher test; *:significantly different at $p<0.05$; **:significantly different at $p<0.01$.

30 Figures 11A and 11B. Maximal strength exercised with four limbs (11a) or only forelimbs (11b) measured in treated or control mice. Results are expressed in sec, as means \pm SEM. Post-hoc comparison to vehicle group was performed with Fisher test; *:significantly different at $p<0.05$; **:significantly different at $p<0.01$; ***:significantly different at $p<0.001$.

Figure 12. Graph of motor neuron velocity in normal and Dhh^{-/-} mice

Figures 13A and 13B. Micrographs of peripheral nerve cells in normal and Dhh^{-/-} mice.

Figures 14A and 14B. Immunohistochemical stains of peripheral nerves using
5 antibodies for neurofilament (an axonal marker) and Laminin (and ECM/connective
tissue marker).

Figure 15. Effects of hedgehog on perineural cell proliferation.

Figure 16. Running time (walking test) in control and treated mice.

Figure 17. Time before falling from the rotarod in control and treated mice.

10 Figure 18. Histological study of SOD mice treated with 500 µg/kg SHH.
Motoneurons were counted in ventral horns of lumbar spinal cord sections originating
from 100 day-old hSOD mice, after cresyl violet staining.

Figure 19. Histological study of SOD mice treated with 500 µg/kg SHH (without
Y0 littermate).

15 Figure 20. Histological study of male SOD mice treated with 500 µg/kg SHH.

Figure 21. Histological study of female SOD mice treated with 500 µg/kg SHH

Figure 22. Evaluating the effect of Hedgehog proteins on ability to grip
following sciatic nerve crush injury.

20 Figure 23. Evaluating the effect of Hedgehog protein on sensory nerve
conduction velocity in galactose intoxication-mediated neuropathies. CA= normal
animal treated with control; CB= normal animal treated with Shh; GA= galactose
intoxicated animal treated with vehicle; and GB= galactose intoxicated animal treated
with Shh.

25 *Detailed Description of the Invention*

The Peripheral Nervous System is one of the two main divisions of the body's
nervous system. The other is the Central Nervous System, which includes the brain and
spinal cord. "Peripheral" means away from the center: and this system contains the
nerves that connect the Central Nervous System to the muscles, skin and internal organs.

30 Peripheral Neuropathy is the term used to describe disorders resulting from
injury (e.g., mechanical, chemical, viral, bacterial or genetic) to the peripheral nerves. It
can be caused by diseases that affect only the peripheral nerves or by conditions that

affect other parts of the body as well. Dymptoms almost always involve weakness, numbness or pain - usually in the arms and legs. It will be helpful for you to know a few basics of nerve biology to understand how neuropathy gets started.

5 I. Overview

 The present application is directed to the discovery that hedgehog gene products are able to protect peripheral nerve cells under conditions which otherwise result in peripheral neuropathy. Certain aspects of the invention are directed to preparations of hedgehog polypeptides, or other molecules which regulate *patched* or *smoothened* signalling, and their uses as protective agents against both acquired and hereditary neuropathies. As used herein, "peripheral neuropathy" refers to a disorder affecting a segment of the peripheral nervous system. For instance, the method of the present invention can be used as part of a treatment program in the management of neuropathies associated with systemic disease, e.g., viral infections, diabetes, inflammation; as well as
10 genetically acquired (hereditary) neuropathies, e.g., Charcot-Marie-Tooth disease; and
15 neuropathies caused by a toxic agent, e.g., a chemotherapeutic agent such as vincristine.

 To further illustrate, the subject method can be used in the treatment of such acquired neuropathies as diabetic neuropathies; immune-mediated neuropathies such as Guillain-Barre syndrome (GBS) and variants, chronic inflammatory demyelinating
20 polyneuropathy (CIDP); chronic polyneuropathies with antibodies to peripheral nerves, neuropathies associated with vasculitis or inflammation of the blood vessels in peripheral nerve, brachial or lumbosacral plexitis, and neuropathies associated with monoclonal gammopathies; neuropathies associated with tumors or neoplasms such as sensory neuropathy associated with lung cancer, neuropathy associated with multiple
25 myeloma, neuropathy associated with waldenstrom's macroglobulemia, chronic lymphocytic leukemia, or B-cell lymphoma; neuropathy associated with amyloidosis; neuropathies caused by infections; neuropathies caused by nutritional imbalance; neuropathy in kidney disease; hypothyroid neuropathy; neuropathy caused by alcohol and toxins; neuropathies caused by drugs; neuropathy resulting from local irradiation;
30 neuropathies caused by trauma or compression; idiopathic neuropathies

 Likewise, the subject method can be used in the treatment of such hereditary neuropathies as Charcot-Marie Tooth Disease (CMT); Familial Amyloidotic Neuropathy and other Hereditary Neuropathies; and Hereditary Porphyria.

 In another embodiment, the subject method can be used to inhibit or otherwise
35 slow neurodegenerative events associated with age-related neuropathology.

As described in the appended examples, *hedgehog* proteins are neuroprotective under conditions which promote chemical lesioning of peripheral nerves. Indeed, *hedgehog* proteins showed a significant protective effective that was similar to the reported effect of NGF. Based upon its neurotrophic and neuroprotective activities, the administration of *hedgehog* or *ptc* therapeutics is suggested herein as a treatment for several types of neurodegenerative diseases including neuropathies. In general, the method of the present invention comprises administering to animal, or to cultured peripheral nerves in vitro, an amount of a *hedgehog* or *ptc* therapeutic (defined *infra*) which produces a non-toxic response by the cell of resistance to degeneration, e.g., marked by loss of differentiation, apoptosis and/or necrosis. The subject method can be carried out on cells which may be either dispersed in culture or a part of an intact tissue or organ. Moreover, the method can be performed on cells which are provided in culture (*in vitro*), or on cells in a whole animal (*in vivo*).

In one aspect, the present invention provides pharmaceutical preparations and methods for treating or preventing neuropathies utilizing, as an active ingredient, a *hedgehog* polypeptide or a mimetic thereof. The invention also relates to methods of controlling the functional performance of peripheral nerve cells by use of the pharmaceutical preparations of the invention.

The subject *hedgehog* treatments are effective on both human and animal subjects afflicted with these conditions. Animal subjects to which the invention is applicable extend to both domestic animals and livestock, raised either as pets or for commercial purposes. Examples are dogs, cats, cattle, horses, sheep, hogs and goats.

Without wishing to be bound by any particular theory, the neuroprotective effect of *hedgehog* treatment may be due at least in part to the ability of these proteins to antagonize (directly or indirectly) *patched*-mediated regulation of gene expression and other physiological effects mediated by that protein. The *patched* gene product, a cell surface protein, is understood to signal through a pathway which causes transcriptional repression of members of the Wnt and Dpp/BMP families of morphogens, proteins which impart positional information. In development of the CNS and patterning of limbs in vertebrates, the introduction of *hedgehog* relieves (derepresses) this inhibition conferred by *patched*, allowing expression of particular gene programs.

Recently, it has been reported that mutations in the human version of *patched*, a gene first identified in a fruit fly developmental pathway, cause a hereditary skin cancer and may contribute to sporadic skin cancers. See, for example, Hahn et al. (1996) *Cell* 86:841-851; and Johnson et al. (1996) *Science* 272:1668-1671. The demonstration that nevoid basal-cell carcinoma (NBCC) results from mutations in the human *patched* gene

provided an example of the roles *patched* plays in post-embryonic development. These observations have led the art to understand one activity of *patched* to be a tumor suppressor gene, which may act by inhibiting proliferative signals from *hedgehog*. Our observations set forth below reveal potential new roles for the *hedgehog/patched* pathway in maintenance of peripheral nerve cells. Accordingly, the present invention contemplates the use of other agents which are capable of mimicking the effect of the *hedgehog* protein on *patched* signalling, e.g., as may be identified from the drug screening assays described below.

In still other embodiments, antagonists of the *hedgehog* signaling can be used in the selective ablation of sensory neurons, for example, in the treatment of chronic pain syndromes.

II. Definitions

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

The term "hedgehog therapeutic" refers to various forms of hedgehog polypeptides, as well as peptidomimetics, which can modulate the proliferation/differentiation state of peripheral nerve cells by, as will be clear from the context of individual examples, mimicking or potentiating (agonizing) or inhibiting (antagonizing) the effects of a naturally-occurring *hedgehog* protein. A *hedgehog* therapeutic which mimics or potentiates the activity of a wild-type hedgehog protein is a "hedgehog agonist". Conversely, a *hedgehog* therapeutic which inhibits the activity of a wild-type hedgehog protein is a "hedgehog antagonist".

In particular, the term "hedgehog polypeptide" encompasses preparations of *hedgehog* proteins and peptidyl fragments thereof, both agonist and antagonist forms as the specific context will make clear.

As used herein the term "bioactive fragment of a *hedgehog* protein" refers to a fragment of a full-length *hedgehog* polypeptide, wherein the fragment specifically agonizes or antagonizes inductive events mediated by wild-type *hedgehog* proteins. The *hedgehog* bioactive fragment preferably is a soluble extracellular portion of a *hedgehog* protein, where solubility is with reference to physiologically compatible solutions. Exemplary bioactive fragments are described in PCT publications WO 95/18856 and WO 96/17924.

The term "ptc therapeutic" refers to agents which either (i) mimic the effect of *hedgehog* proteins on *patched* signalling, e.g., which antagonize the cell-cycle inhibitory activity of *patched*, or (ii) activate or potentiate *patched* signalling. In other embodiments, the ptc therapeutic can be a *hedgehog* antagonist. The ptc therapeutic can be, e.g., a peptide, a nucleic acid, a carbohydrate, a small organic molecule, or natural product extract (or fraction thereof).

An "effective amount" of, e.g., a hedgehog therapeutic, with respect to the subject method of treatment, refers to an amount of, e.g., a hedgehog polypeptide in a preparation which, when applied as part of a desired dosage regimen brings enhances the survival of peripheral nerves, relative to the absence of the hedgehog therapeutic, according to clinically acceptable standards for the disorder to be treated.

A "patient" or "subject" to be treated by the subject method can mean either a human or non-human animal.

The "growth state" of a cell refers to the rate of proliferation of the cell and the state of differentiation of the cell.

"Homology" and "identity" each refer to sequence similarity between two polypeptide sequences, with identity being a more strict comparison. Homology and identity can each be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same amino acid residue, then the polypeptides can be referred to as identical at that position; when the equivalent site is occupied by the same amino acid (e.g., identical) or a similar amino acid (e.g., similar in steric and/or electronic nature), then the molecules can be referred to as homologous at that position. A percentage of homology or identity between sequences is a function of the number of matching or homologous positions shared by the sequences. An "unrelated" or "non-homologous" sequence shares less than 40 percent identity, though preferably less than 25 percent identity, with an hedgeog sequence of the present invention.

The term "corresponds to", when referring to a particular polypeptide or nucleic acid sequence is meant to indicate that the sequence of interest is identical or homologous to the reference sequence to which it is said to correspond.

The terms "recombinant protein", "heterologous protein" and "exogenous protein" are used interchangeably throughout the specification and refer to a polypeptide which is produced by recombinant DNA techniques, wherein generally, DNA encoding the polypeptide is inserted into a suitable expression construct which is in turn used to

transform a host cell to produce the heterologous protein. That is, the polypeptide is expressed from a heterologous nucleic acid.

5 A "chimeric protein" or "fusion protein" is a fusion of a first amino acid sequence encoding a *hedgehog* polypeptide with a second amino acid sequence defining a domain foreign to and not substantially homologous with any domain of *hh* protein. A chimeric protein may present a foreign domain which is found (albeit in a different protein) in an organism which also expresses the first protein, or it may be an "interspecies", "intergenic", etc. fusion of protein structures expressed by different kinds of organisms. In general, a fusion protein can be represented by the general formula
10 $(X)_n-(hh)_m-(Y)_n$, wherein *hh* represents all or a portion of the *hedgehog* protein, X and Y each independently represent an amino acid sequences which are not naturally found as a polypeptide chain contiguous with the *hedgehog* sequence, m is an integer greater than or equal to 1, and each occurrence of n is, independently, 0 or an integer greater than or equal to 1 (n and m are preferably no greater than 5 or 10).

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III. Exemplary Applications of Method and Compositions

The subject method has wide applicability to the treatment or prophylaxis of disorders affecting the regulation of peripheral nerves, including peripheral ganglionic neurons, sympathetic, sensory neurons, and motor neurons. In general, the method can
20 be characterized as including a step of administering to an animal an amount of a *ptc* or *hedgehog* therapeutic effective to alter the proliferative and/or differentiation state of treated peripheral nerve cells. Such therapeutic compositions may be useful in treatments designed to rescue, for example, retinal ganglia, inner ear and accoustical nerves, and motoneurons, from lesion-induced death as well as guiding reprojection of
25 these neurons after such damage. Such diseases and conditions include, but are not limited to, chemical or mechanical trauma, infection (such as viral infection with varicella-zoster), metabolic disease such as diabetes, nutritional deficiency, toxic agents (such as cisplatin treatment). The goals of treatment in each case can be twofold: (1) to eliminate the cause of the disease and (2) to relieve its symptoms.

30 Peripheral neuropathy is a condition involving nerve-ending damage in the hands and feet. Peripheral neuropathy generally refers to a disorder that affects the peripheral nerves, most often manifested as one or a combination of motor, sensory, sensorimotor, or autonomic neural dysfunction. The wide variety of morphologies exhibited by peripheral neuropathies can each be uniquely attributed to an equally wide
35 variety of causes. For instance, peripheral neuropathies can be genetically acquired, can

result from a systemic disease, or can be induced by a toxic agent. Some toxic agents that cause neurotoxicities are therapeutic drugs, antineoplastic agents, contaminants in foods or medicinals, and environmental and industrial pollutants.

5 In particular, chemotherapeutic agents known to cause sensory and/or motor neuropathies include vincristine, an antineoplastic drug used to treat haematological malignancies and sarcomas, as well as cisplatin, taxol and others. The neurotoxicity is dose-related, and exhibits as reduced intestinal motility and peripheral neuropathy, especially in the distal muscles of the hands and feet, postural hypotension, and atony of the urinary bladder. Similar problems have been documented with taxol and cisplatin
10 (Mollman, J. E., 1990, New Eng Jour Med. 322:126-127), although cisplatin-related neurotoxicity can be alleviated with nerve growth factor (NGF) (Apfel, S. C. et al, 1992, Annals of Neurology 31:76-80). Although the neurotoxicity is sometimes reversible after removal of the neurotoxic agent, recovery can be a very slow process (Legha, S., 1986, Medical Toxicology 1:421-427; Olesen, et al., 1991, Drug Safety 6:302-314).

15 There are a number of inherited peripheral neuropathies, including: Refsum's disease, Abetalipoproteinemia, Tangier disease, Krabbe's disease, Metachromatic leukodystrophy, Fabry's disease, Dejerine-Sottas syndrome, and others. Of all the inherited neuropathies, the most common by far is Charcot-Marie-Tooth Disease.

Charcot-Marie-Tooth (CMT) Disease (also known as Peroneal Muscular
20 Atrophy, or Hereditary Motor Sensory Neuropathy (HMSN)) is the most common hereditary neurological disorder. It is characterized by weakness and atrophy, primarily of the peroneal muscles, due to segmental demyelination of peripheral nerves and associated degeneration of axons and anterior horn cells. Autosomal dominant inheritance is usual, and associated degenerative CNS disorders, such as Friedreich's
25 ataxia, are common.

In one aspect, the method of the present invention can be used in the treatment and maintenance of hereditary neuropathies. This group of neuropathies are now becoming increasingly recognized due to the dramatic advances in molecular genetics. The symptoms of the various hereditary neuropathies are wide ranging. A common
30 denominator is usually the early onset of mild numbness and tingling in the feet that slowly progresses to involve the legs and the hands and later the rest of the upper extremities. Most of the hereditary neuropathies do have a motor component consisting of distal weakness in the lower and upper extremities. A majority of patients with hereditary neuropathies have high arches in their feet or other bony deformities. The
35 symptoms are very slowly progressive and the majority of the patients are still walking two decades after the onset of their symptoms.

The diagnosis of a hereditary neuropathy is usually suggested with the early onset of neuropathic symptoms, especially when a positive family history is also present. Prior to the recent genetic advances, the diagnosis was supported by typical findings of marked slowing of the nerve conduction studies on electromyography and a nerve biopsy. Typical findings on a nerve biopsy include the presence of so-called onion-bulbs, indicating a recurring demyelinating and remyelinating of the nerve fibers. With the most recent genetic advances, two major hereditary neuropathies known as "Charcot-Marie-Tooth disease" and "hereditary neuropathy with liability to pressure palsies" can be diagnosed with a simple blood test that identifies the different mutations responsible for these two entities.

Hereditary neuropathies are caused by genetic abnormalities which are transmitted from generation to generation. For several of these, the genetic defect is known, and tests are available for diagnosis and prenatal counseling.

As set forth above, the subject method can be used as part of a therapeutic regimen in the treatment of Charcot-Marie Tooth Disease (CMT). This is a general term given to the hereditary sensorimotor neuropathies. CMT type 1 (CMT 1) is associated with demyelination or breakdown of the myelin sheaths. Several different abnormalities have been identified. CMT Type 1A is most commonly caused by duplication of a gene encoding a myelin protein called PMP-22, and CMT type 1B is caused by a mutation in a myelin protein called the Po glycoprotein. CMTX is a hereditary sensorimotor neuropathy which affects only men. It is caused by a mutation in a gene encoding a protein called Connexin 32 on the X-chromosome.

In certain embodiments, the subject method can be used to treat, or at least reduce the severity of, Amyotrophic lateral sclerosis (ALS). According to the subject invention, a trophic amount of a *hedgehog* or *ptc* therapeutic can be administered to an animal suffering from, or at risk of developing, ALS.

In another embodiment, the subject method can be used in the treatment of Familial Amyloidotic Neuropathy and other related hereditary neuropathies. Amyloidotic neuropathy usually presents with pain, sensory loss and autonomic dysfunction. It is caused by a mutation in a protein called Transthyretin, resulting in deposition of the protein as amyloid in the peripheral nerves.

The subject method can be used in the treatment of hereditary porphyria, which can have components of peripheral neuropathy.

Still another hereditary neuropathy for which the subject methods can be used for treatment is hereditary sensory neuropathy Type II (HSN II).

The methods and compositions of the present invention can also be used in the treatment and maintenance of acquired neuropathies.

For example, hedgehog and ptc therapeutics can be used to prevent diabetic neuropathies. Diabetes is the most common known cause of neuropathy. It produces
5 symptoms in approximately 10% of people with diabetes. In most cases, the neuropathy is predominantly sensory, with pain and sensory loss in the hands and feet. But some diabetics have mononeuritis or mononeuritis multiplex which causes weakness in one or more nerves, or lumbosacral plexopathy or amyotrophy which causes weakness in the legs.

10 The instant method can also be used in the treatment of immune-mediated neuropathies. The main function of the immune system is to protect the body against infectious organisms which enter from outside. In some cases, however the immune system turns against the body and causes autoimmune disease. The immune system consists of several types of white blood cells, including T-lymphocytes, which also
15 regulate the immune response; and B-lymphocytes or plasma cells, which secrete specialized proteins called "antibodies" Sometimes, for unknown reasons, the immune system mistakenly attacks parts of the body such as the peripheral nerves. This is "autoimmune" Peripheral Neuropathy. There are several different types, depending on the part of the peripheral nerve which is attacked and the type of the immune reaction.
20 The following are brief descriptions of the neuropathies which are mediated by the immune system.

For instance, a hedgehog or ptc therapeutic can be used to treat Guillain-Barre Syndrome (GBS). An acute neuropathy because it comes on suddenly or rapidly. Guillain-Barre Syndrome can progress to paralysis and respiratory failure within days or
25 weeks after onset. The neuropathy is caused when the immune system destroys the myelin sheaths of the motor and sensory nerves. It is often preceded by infection, vaccination or trauma, and that is thought to be what triggers the autoimmune reaction. The disease is self-limiting, with spontaneous recovery within six to eight weeks. But the recovery is often incomplete.

30 Other neuropathies which begin acutely, and which can be treated by the method of the present invention, include Acute Motor Neuropathy, Acute Sensory Neuropathy, and Acute Autonomic Neuropathy, in which there is an immune attack against the motor, sensory or autonomic nerves, respectively. The Miller-Fisher Syndrome is another variant in which there is paralysis of eye gaze, incoordination, and unsteady gait

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Still another acquired neuropathy which is may be treated by the subject method is Chronic Inflammatory Demyelinating Polyneuropathy (CIDP). CIDP is thought to be a chronic and more indolent form of the Guillain-Barre Syndrome. The disease progresses either with repeated attacks, called relapses, or in a stepwise or steady fashion. As in GBS, there appears to be destruction of the myelin sheath by antibodies and T-lymphocytes. But since there is no specific test for CIDP, the diagnosis is based on the clinical and laboratory characteristics.

Chronic Polyneuropathies with antibodies to peripheral nerves is still another peripheral neuropathy for which the subject methods can be employed to treat or prevent. In some types of chronic neuropathies, antibodies to specific components of nerve have been identified. These include demyelinating neuropathy associated with antibodies to the Myelin Associated Glycoprotein (MAG), motor neuropathy associated with antibodies to the gangliosides GM1 or GD1a, and sensory neuropathy associated with anti-sulfatide or GD1b ganglioside antibodies. The antibodies in these cases bind to oligosaccharide or sugar like molecules, which are linked to proteins (glycoproteins) or lipids (glycolipids or gangliosides) in the nerves. It is suspected that these antibodies may be responsible for the neuropathies.

The subject method can also be used as part of a therapeutic plan for treating neuropathies associated with vasculitis or inflammation of the blood vessels in peripheral nerves. Neuropathy can also be caused by Vasculitis - an inflammation of the blood vessels in peripheral nerve. It produces small "strokes" along the course of the peripheral nerves, and may be restricted to the nerves or it may be generalized, include a skin rash, or involve other organs. Several rheumatological diseases like Rheumatoid Arthritis, Lupus, Periarthritis Nodosa, or Sjogren's Syndrome, are associated with generalized Vasculitis, which can also involve the peripheral nerves. Vasculitis can cause Polyneuritis, Mononeuritis, or Mononeuritis Multiplex, depending on the distribution and severity of the lesions.

In still another embodiment, the method of the present invention can be used for treatment of brachial or lumbosacral plexitis. The brachial plexus, which lies under the armpit, contains the nerves to the arm and hand. Brachial Plexitis is the result of inflammation of that nerve bundle, and produces weakness and pain in one or both arms. Lumbosacral Plexitis, which occurs in the pelvis, causes weakness and pain in the legs.

Hedgehog and ptc therapeutics mayu also be suitable for use in the treatment of neuropathies associated with monoclonal gammopathies. In Monoclonal Gammopathy, single clones of B-cells or plasma cells in the bone marrow or lymphoid organs expand to form benign or malignant tumors and secrete antibodies. "Monoclonal" is because

there are single clones of antibodies. And "Gammopathy" stands for gammaglobulins, which is another name for antibodies. In some cases, the antibodies react with nerve components; in others, fragments of the antibodies form amyloid deposits.

Yet another aspect of the present invention relates to the use of the subject
5 method in the treatment of neuropathies associated with tumors or neoplasms. Neuropathy can be due to direct infiltration of nerves by tumor cells or to indirect effect of the tumor. The latter is called Paraneoplastic Neuropathy. Several types have been described. For instance, the subject methods can be used to manage sensory neuropathy associated with lung cancer. This neuropathy is associated with antibodies to a protein
10 called Hu, which is present in the sensory neurons of the peripheral nerves. Likewise, the subject method can be used to treat neuropathies associated with multiple myeloma. Multiple myeloma is a bony tumor which is caused by antibody-secreting plasma cells in the bone marrow. The tumor is made up of a single clone of plasma cells, and the antibodies they produce are identical or monoclonal. Some people with multiple
15 myeloma develop a Sensorimotor Polyneuropathy with degeneration of axons in the peripheral nerves. In other embodiments, the subject method can be used to treat neuropathies associated with Waldenstrom's Macroglobulemia, Chronic Lymphocytic Leukemia, or B-cell Lymphoma. These are tumors caused by antibody-secreting B-lymphocytes in the spleen, bone marrow or lymph nodes. These antibodies are
20 monoclonal and frequently react with peripheral nerve components such as MAG, GM1, or sulfatide. In still other embodiments, the the hedgehog and ptc therapeutics of the present invention can be used as part of therapeutic protocol for the treatment of patients with cancers where neuropathy is a consequence of local irradiation or be caused by medications such as vincristine and cisplatinum.

25 The present invention also contemplates the use of hedgehog and ptc therapeutics for the treatment of neuropathies associated with amyloidosis. Amyloid is a substance which is deposited in the peripheral nerves and interferes with their operation: the disorder is Amyloidosis. There are two main types: Primary Amyloidosis, in which the deposits contain fragments of monoclonal antibodies (see the Monoclonal Gammopathy
30 paragraph above); and Hereditary Amyloidosis in which the deposits contain a mutated protein called Transthyretin. Primary Amyloidosis is usually associated with Monoclonal Gammopathies or myeloma (See above.)

Still another aspect of the present invention provides the subject method as a means for treating neuropathies caused by infections. Peripheral neuropathies can be
35 caused by infection of the peripheral nerves. Viruses that cause peripheral neuropathies include the AIDS virus, HIV-I, which causes slowly progressive sensory neuropathy,

Cytomegalo virus which causes a rapidly progressive paralytic neuropathy, Herpes Zoster which cause Shingles, and Poliovirus which causes a motor neuropathy. Hepatitis B or C infections are sometimes associated with vasculitic neuropathy.

5 Bacterial infections that cause neuropathy include Leprosy which causes a patchy sensory neuropathy, and Diphtheria which can cause a rapidly progressive paralytic neuropathy. Other infectious diseases that cause neuropathy include Lyme disease which is caused by a spirochete, and Trypanosomiasis which is caused by a parasite. Both commonly present with a multifocal neuropathy

10 Neuropathies caused by nutritional imbalance are also candidate disorders for treatment by the subject method. Deficiencies of Vitamins B12, B1 (thiamine), B6 (pyridoxine), or E, for example, can produce polyneuropathies with degeneration of peripheral nerve axons. This can be due to poor diet, or inability to absorb the nutrients from the stomach or gut.

15 Moreover megadoses of Vitamin B6 can also cause a peripheral neuropathy, and the subject method can be used as part of a de-toxification program in such cases.

Yet another use of the subject method is in the treatment of neuropathies arising in kidney diseases. Chronic renal failure can cause a predominantly sensory peripheral neuropathy with degeneration of peripheral nerve axons.

20 Another aspect of the present invention provides a method for treating hypothyroid neuropathies. Hypothyroidism is sometimes associated with a painful sensory polyneuropathy with axonal degeneration. Mononeuropathy or Mononeuropathy Multiplex can also occur due to compression of the peripheral nerves by swollen tissues.

25 The subject method can also be used in the treatment of neuropathies caused by Alcohol and Toxins. Certain toxins can cause Peripheral Neuropathy. Lead toxicity is associated with a motor neuropathy; arsenic or mercury cause a sensory neuropathy, Thalium can cause a sensory and autonomic neuropathy. several of the organic solvents and insecticides can also cause polyneuropathy. Alcohol is directly toxic to nerves and alcohol abuse is a major cause of neuropathy. The subject method can be used, in certain embodiments, as part of a broader detoxification program.

30 In still another embodiment, the methods and compositions of the present invention can be used for the treatment of neuropathies caused by drugs. Several drugs are known to cause neuropathy. They include, among others, vincristine and cisplatin in cancer, nitrofurantoin, which is used in pyelonephritis, amiodarone in cardiac arrhythmias, disulfiram in alcoholism, ddC and ddI in AIDS, and dapsone which is used

to treat Leprosy. As above, the subject method can be used, in certain embodiments, as part of a broader detoxification program.

The method of the present invention can also be used in the treatment of neuropathies caused by trauma or compression. Localized neuropathies can result from
5 compression of nerves by external pressure or overlying tendons and other tissues. The best known of these are the Carpal Tunnel Syndrome which results from compression at the wrist, and cervical or lumbar radiculopathies (Sciatica) which result from compression of nerve roots as they exit the spine. Other common areas of nerve compression include the elbows, armpits, and the back of the knees.

10 The subject method is also useful in variety of idiopathic neuropathies. The term "idiopathic" is used whenever the cause of the neuropathy cannot be found. In these cases, the neuropathy is classified according to its manifestations, i.e., sensory, motor, or sensorimotor idiopathic polyneuropathy.

Another aspect of the invention provides a conjoint therapy wherein one or more
15 other therapeutic agents are administered with the hedgehog or ptc therapeutic agent. Such conjoint treatment may be achieved by way of the simultaneous, sequential or separate dosing of the individual components of the treatment. For example, the subject method can be carried out conjointly with other neuroprotective agents. The dosages recited herein would be adjusted to compensate for such additional components in the
20 therapeutic composition. Progress of the treated patient can be monitored by conventional methods.

IV. Exemplary hedgehog therapeutic compounds.

The *hedgehog* therapeutic compositions of the subject method can be generated
25 by any of a variety of techniques, including purification of naturally occurring proteins, recombinantly produced proteins and synthetic chemistry. Polypeptide forms of the hedgehog therapeutics are preferably derived from vertebrate hedgehog proteins, e.g., have sequences corresponding to naturally occurring hedgehog proteins, or fragments thereof, from vertebrate organisms. However, it will be appreciated that the hedgehog
30 polypeptide can correspond to a hedgehog protein (or fragment thereof) which occurs in any metazoan organism.

The various naturally-occurring *hedgehog* proteins from which the subject therapeutics can be derived are characterized by a signal peptide, a highly conserved N-terminal region, and a more divergent C-terminal domain. In addition to signal sequence
35 cleavage in the secretory pathway (Lee, J.J. *et al.* (1992) *Cell* 71:33-50; Tabata, T. *et al.*

(1992) *Genes Dev.* 2635-2645; Chang, D.E. *et al.* (1994) *Development* 120:3339-3353), *hedgehog* precursor proteins naturally undergo an internal autoproteolytic cleavage which depends on conserved sequences in the C-terminal portion (Lee *et al.* (1994) *Science* 266:1528-1537; Porter *et al.* (1995) *Nature* 374:363-366). This autocleavage leads to a 19 kD N-terminal peptide and a C-terminal peptide of 26-28 kD (Lee *et al.* (1992) *supra*; Tabata *et al.* (1992) *supra*; Chang *et al.* (1994) *supra*; Lee *et al.* (1994) *supra*; Bumcrot, D.A., *et al.* (1995) *Mol. Cell. Biol.* 15:2294-2303; Porter *et al.* (1995) *supra*; Ekker, S.C. *et al.* (1995) *Curr. Biol.* 5:944-955; Lai, C.J. *et al.* (1995) *Development* 121:2349-2360). The N-terminal peptide stays tightly associated with the surface of cells in which it was synthesized, while the C-terminal peptide is freely diffusible both *in vitro* and *in vivo* (Lee *et al.* (1994) *supra*; Bumcrot *et al.* (1995) *supra*; Mart', E. *et al.* (1995) *Development* 121:2537-2547; Roelink, H. *et al.* (1995) *Cell* 81:445-455). Cell surface retention of the N-terminal peptide is dependent on autocleavage, as a truncated form of *hedgehog* encoded by an RNA which terminates precisely at the normal position of internal cleavage is diffusible *in vitro* (Porter *et al.* (1995) *supra*) and *in vivo* (Porter, J.A. *et al.* (1996) *Cell* 86, 21-34). Biochemical studies have shown that the autoproteolytic cleavage of the *hedgehog* precursor protein proceeds through an internal thioester intermediate which subsequently is cleaved in a nucleophilic substitution. It is suggested that the nucleophile is a small lipophilic molecule, more particularly cholesterol, which becomes covalently bound to the C-terminal end of the N-peptide (Porter *et al.* (1996) *supra*), tethering it to the cell surface.

The vertebrate family of *hedgehog* genes includes at least four members, e.g., paralogs of the single drosophila *hedgehog* gene (SEQ ID No. 19). Three of these members, herein referred to as Desert *hedgehog* (*Dhh*), Sonic *hedgehog* (*Shh*) and Indian *hedgehog* (*Ihh*), apparently exist in all vertebrates, including fish, birds, and mammals. A fourth member, herein referred to as tiggie-winkle *hedgehog* (*Thh*), appears specific to fish. According to the appended sequence listing, (see also Table 1) a chicken *Shh* polypeptide is encoded by SEQ ID No:1; a mouse *Dhh* polypeptide is encoded by SEQ ID No:2; a mouse *Ihh* polypeptide is encoded by SEQ ID No:3; a mouse *Shh* polypeptide is encoded by SEQ ID No:4 a zebrafish *Shh* polypeptide is encoded by SEQ ID No:5; a human *Shh* polypeptide is encoded by SEQ ID No:6; a human *Ihh* polypeptide is encoded by SEQ ID No:7; a human *Dhh* polypeptide is encoded by SEQ ID No. 8; and a zebrafish *Thh* is encoded by SEQ ID No. 9.

35

Table 1
Guide to *hedgehog* sequences in Sequence Listing

Nucleotide	Amino Acid
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Chicken <i>Shh</i>	SEQ ID No. 1	SEQ ID No. 10
Mouse <i>Dhh</i>	SEQ ID No. 2	SEQ ID No. 11
Mouse <i>Ihh</i>	SEQ ID No. 3	SEQ ID No. 12
Mouse <i>Shh</i>	SEQ ID No. 4	SEQ ID No. 13
Zebrafish <i>Shh</i>	SEQ ID No. 5	SEQ ID No. 14
Human <i>Shh</i>	SEQ ID No. 6	SEQ ID No. 15
Human <i>Ihh</i>	SEQ ID No. 7	SEQ ID No. 16
Human <i>Dhh</i>	SEQ ID No. 8	SEQ ID No. 17
Zebrafish <i>Thh</i>	SEQ ID No. 9	SEQ ID No. 18
Drosophila <i>HH</i>	SEQ ID No. 19	SEQ ID No. 20

In addition to the sequence variation between the various *hedgehog* homologs, the *hedgehog* proteins are apparently present naturally in a number of different forms, including a pro-form, a full-length mature form, and several processed fragments thereof. The pro-form includes an N-terminal signal peptide for directed secretion of the extracellular domain, while the full-length mature form lacks this signal sequence.

As described above, further processing of the mature form occurs in some instances to yield biologically active fragments of the protein. For instance, *sonic hedgehog* undergoes additional proteolytic processing to yield two peptides of approximately 19 kDa and 27 kDa, the 19kDa fragment corresponding to an proteolytic N-terminal portion of the mature protein.

In addition to proteolytic fragmentation, the vertebrate *hedgehog* proteins can also be modified post-translationally, such as by glycosylation and/or addition of lipophilic moieties, such as stents, fatty acids, etc., though bacterially produced (e.g. unmodified) forms of the proteins still maintain certain of the bioactivities of the native protein. Bioactive fragments of *hedgehog* polypeptides of the present invention have been generated and are described in great detail in, e.g., PCT publications WO 95/18856 and WO 96/17924.

There are a wide range of lipophilic moieties with which *hedgehog* polypeptides can be derivatized. The term "lipophilic group", in the context of being attached to a *hedgehog* polypeptide, refers to a group having high hydrocarbon content thereby giving the group high affinity to lipid phases. A lipophilic group can be, for example, a relatively long chain alkyl or cycloalkyl (preferably n-alkyl) group having approximately 7 to 30 carbons. The alkyl group may terminate with a hydroxy or primary amine "tail". To further illustrate, lipophilic molecules include naturally-occurring and synthetic aromatic and non-aromatic moieties such as fatty acids, sterols, esters and alcohols, other lipid molecules, cage structures such as adamantane and

buckminsterfullerenes, and aromatic hydrocarbons such as benzene, perylene, phenanthrene, anthracene, naphthalene, pyrene, chrysene, and naphthacene.

In one embodiment, the hedgehog polypeptide is modified with one or more sterol moieties, such as cholesterol. See, for example, PCT publication WO 96/17924.
5 In certain embodiments, the cholesterol is preferably added to the C-terminal glycine where the hedgehog polypeptide corresponds to the naturally-occurring N-terminal proteolytic fragment.

In another embodiment, the hedgehog polypeptide can be modified with a fatty acid moiety, such as a myristoyl, palmitoyl, stearyl, or arachidoyl moiety. See, e.g.,
10 Pepinsky et al. (1998) J Biol. Chem 273: 14037.

In addition to those effects seen by cholesterol-addition to the C-terminus or fatty acid addition to the N-terminus of extracellular fragments of the protein, at least certain of the biological activities of the hedgehog gene products are unexpectedly potentiated by derivatization of the protein with lipophilic moieties at other sites on the protein
15 and/or by moieties other than cholesterol or fatty acids. Certain aspects of the invention are directed to the use of preparations of hedgehog polypeptides which are modified at sites other than N-terminal or C-terminal residues of the natural processed form of the protein, and/or which are modified at such terminal residues with lipophilic moieties other than a sterol at the C-terminus or fatty acid at the N-terminus.

20 Particularly useful as lipophilic molecules are alicyclic hydrocarbons, saturated and unsaturated fatty acids and other lipid and phospholipid moieties, waxes, cholesterol, isoprenoids, terpenes and polyalicyclic hydrocarbons including adamantane and buckminsterfullerenes, vitamins, polyethylene glycol or oligoethylene glycol, (C1-C18)-alkyl phosphate diesters, -O-CH₂-CH(OH)-O-(C12-C18)-alkyl, and in particular
25 conjugates with pyrene derivatives. The lipophilic moiety can be a lipophilic dye suitable for use in the invention include, but are not limited to, diphenylhexatriene, Nile Red, N-phenyl-1-naphthylamine, Prodan, Laurodan, Pyrene, Perylene, rhodamine, rhodamine B, tetramethylrhodamine, Texas Red, sulforhodamine, 1,1'-didodecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, octadecyl rhodamine B and the
30 BODIPY dyes available from Molecular Probes Inc.

Other exemplary lipophilic moieties include aliphatic carbonyl radical groups include 1- or 2-adamantylacetyl, 3-methyladamant-1-ylacetyl, 3-methyl-3-bromo-1-adamantylacetyl, 1-decalinacetyl, camphoracetyl, camphaneacetyl, noradamantylacetyl, norbornaneacetyl, bicyclo[2.2.2.]-oct-5-eneacetyl, 1-methoxybicyclo[2.2.2.]-oct-5-ene-
35 2-carbonyl, cis-5-norbornene-endo-2,3-dicarbonyl, 5-norbornen-2-ylacetyl, (1R)-(-)-

myrtentaneacetyl, 2-norbornaneacetyl, anti-3-oxo-tricyclo[2.2.1.0<2,6>]-heptane-7-carbonyl, decanoyl, dodecanoyl, dodecenoyl, tetradecadienoyl, decynoyl or dodecynoyl.

The hedgehog polypeptide can be linked to the hydrophobic moiety in a number of ways including by chemical coupling means, or by genetic engineering.

5 There are a large number of chemical cross-linking agents that are known to those skilled in the art. For the present invention, the preferred cross-linking agents are heterobifunctional cross-linkers, which can be used to link the hedgehog polypeptide and hydrophobic moiety in a stepwise manner. Heterobifunctional cross-linkers provide the ability to design more specific coupling methods for conjugating to proteins, thereby
 10 reducing the occurrences of unwanted side reactions such as homo-protein polymers. A wide variety of heterobifunctional cross-linkers are known in the art. These include: succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), m-Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS); N-succinimidyl (4-iodoacetyl) aminobenzoate (SIAB), succinimidyl 4-(p-maleimidophenyl) butyrate (SMPB), 1-ethyl-
 15 3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC); 4-succinimidylloxycarbonyl- α -methyl- α -(2-pyridyldithio)-tolune (SMPT), N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), succinimidyl 6-[3-(2-pyridyldithio) propionate] hexanoate (LC-SPDP). Those cross-linking agents having N-hydroxysuccinimide moieties can be obtained as the N-hydroxysulfosuccinimide analogs, which generally
 20 have greater water solubility. In addition, those cross-linking agents having disulfide bridges within the linking chain can be synthesized instead as the alkyl derivatives so as to reduce the amount of linker cleavage *in vivo*.

In addition to the heterobifunctional cross-linkers, there exists a number of other cross-linking agents including homobifunctional and photoreactive cross-linkers.
 25 Disuccinimidyl suberate (DSS), bismaleimido hexane (BMH) and dimethylpimelimidate-2 HCl (DMP) are examples of useful homobifunctional cross-linking agents, and bis-[β -(4-azidosalicylamido)ethyl]disulfide (BASED) and N-succinimidyl-6(4'-azido-2'-nitrophenyl- amino)hexanoate (SANPAH) are examples of useful photoreactive cross-linkers for use in this invention. For a recent review of
 30 protein coupling techniques, see Means et al. (1990) *Bioconjugate Chemistry* 1:2-12, incorporated by reference herein.

One particularly useful class of heterobifunctional cross-linkers, included above, contain the primary amine reactive group, N-hydroxysuccinimide (NHS), or its water soluble analog N-hydroxysulfosuccinimide (sulfo-NHS). Primary amines (lysine
 35 epsilon groups) at alkaline pH's are unprotonated and react by nucleophilic attack on

NHS or sulfo-NHS esters. This reaction results in the formation of an amide bond, and release of NHS or sulfo-NHS as a by-product.

Another reactive group useful as part of a heterobifunctional cross-linker is a thiol reactive group. Common thiol reactive groups include maleimides, halogens, and pyridyl disulfides. Maleimides react specifically with free sulfhydryls (cysteine residues) in minutes, under slightly acidic to neutral (pH 6.5-7.5) conditions. Halogens (iodoacetyl functions) react with -SH groups at physiological pH's. Both of these reactive groups result in the formation of stable thioether bonds.

The third component of the heterobifunctional cross-linker is the spacer arm or bridge. The bridge is the structure that connects the two reactive ends. The most apparent attribute of the bridge is its effect on steric hindrance. In some instances, a longer bridge can more easily span the distance necessary to link two complex biomolecules. For instance, SMPB has a span of 14.5 angstroms.

Preparing protein-protein conjugates using heterobifunctional reagents is a two-step process involving the amine reaction and the sulfhydryl reaction. For the first step, the amine reaction, the protein chosen should contain a primary amine. This can be lysine epsilon amines or a primary alpha amine found at the N-terminus of most proteins. The protein should not contain free sulfhydryl groups. In cases where both proteins to be conjugated contain free sulfhydryl groups, one protein can be modified so that all sulfhydryls are blocked using for instance, N-ethylmaleimide (see Partis et al. (1983) J. Pro. Chem. 2:263, incorporated by reference herein). Ellman's Reagent can be used to calculate the quantity of sulfhydryls in a particular protein (see for example Ellman et al. (1958) Arch. Biochem. Biophys. 74:443 and Riddles et al. (1979) Anal. Biochem. 94:75, incorporated by reference herein).

The reaction buffer should be free of extraneous amines and sulfhydryls. The pH of the reaction buffer should be 7.0-7.5. This pH range prevents maleimide groups from reacting with amines, preserving the maleimide group for the second reaction with sulfhydryls.

The NHS-ester containing cross-linkers have limited water solubility. They should be dissolved in a minimal amount of organic solvent (DMF or DMSO) before introducing the cross-linker into the reaction mixture. The cross-linker/solvent forms an emulsion which will allow the reaction to occur.

The sulfo-NHS ester analogs are more water soluble, and can be added directly to the reaction buffer. Buffers of high ionic strength should be avoided, as they have a tendency to "salt out" the sulfo-NHS esters. To avoid loss of reactivity due to

hydrolysis, the cross-linker is added to the reaction mixture immediately after dissolving the protein solution.

The reactions can be more efficient in concentrated protein solutions. The more alkaline the pH of the reaction mixture, the faster the rate of reaction. The rate of hydrolysis of the NHS and sulfo-NHS esters will also increase with increasing pH. Higher temperatures will increase the reaction rates for both hydrolysis and acylation.

Once the reaction is completed, the first protein is now activated, with a sulfhydryl reactive moiety. The activated protein may be isolated from the reaction mixture by simple gel filtration or dialysis. To carry out the second step of the cross-linking, the sulfhydryl reaction, the lipophilic group chosen for reaction with maleimides, activated halogens, or pyridyl disulfides must contain a free sulfhydryl. Alternatively, a primary amine may be modified with to add a sulfhydryl

In all cases, the buffer should be degassed to prevent oxidation of sulfhydryl groups. EDTA may be added to chelate any oxidizing metals that may be present in the buffer. Buffers should be free of any sulfhydryl containing compounds.

Maleimides react specifically with -SH groups at slightly acidic to neutral pH ranges (6.5-7.5). A neutral pH is sufficient for reactions involving halogens and pyridyl disulfides. Under these conditions, maleimides generally react with -SH groups within a matter of minutes. Longer reaction times are required for halogens and pyridyl disulfides.

The first sulfhydryl reactive-protein prepared in the amine reaction step is mixed with the sulfhydryl-containing lipophilic group under the appropriate buffer conditions. The conjugates can be isolated from the reaction mixture by methods such as gel filtration or by dialysis.

Exemplary activated lipophilic moieties for conjugation include: N-(1-pyrene)maleimide; 2,5-dimethoxystilbene-4'-maleimide, eosin-5-maleimide; fluorescein-5-maleimide; N-(4-(6-dimethylamino-2-benzofuranyl)phenyl)maleimide; benzophenone-4-maleimide; 4-dimethylaminophenylazophenyl-4'-maleimide (DABMI), tetramethylrhodamine-5-maleimide, tetramethylrhodamine-6-maleimide, Rhodamine Red™ C2 maleimide, N-(5-aminopentyl)maleimide, trifluoroacetic acid salt, N-(2-aminoethyl)maleimide, trifluoroacetic acid salt, Oregon Green™ 488 maleimide, N-(2-((2-((4-azido-2,3,5,6-tetrafluorobenzoyl)amino)ethyl)dithio)ethyl)maleimide (TFPAM-SS1), 2-(1-(3-dimethylaminopropyl)-indol-3-yl)-3-(indol-3-yl) maleimide (bisindolylmaleimide; GF 109203X), BODIPY® FL N-(2-aminoethyl)maleimide, N-(7-dimethylamino-4-methylcoumarin-3-

yl)maleimide (DACM), AlexaTM 488 C5 maleimide, AlexaTM 594 C5 maleimide, sodium saltN-(1-pyrene)maleimide, 2,5-dimethoxystilbene-4'-maleimide, eosin-5-maleimide, fluorescein-5-maleimide, N-(4-(6-dimethylamino-2-benzofuranyl)phenyl)maleimide, benzophenone-4-maleimide, 4-dimethylaminophenylazophenyl-4'-maleimide, 1-(2-maleimidylethyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl)pyridinium methanesulfonate, tetramethylrhodamine-5-maleimide, tetramethylrhodamine-6-maleimide, Rhodamine RedTM C2 maleimide, N-(5-aminopentyl)maleimide, N-(2-aminoethyl)maleimide, N-(2-((2-(((4-azido-2,3,5,6-tetrafluoro)benzoyl) amino)ethyl)dithio)ethyl)maleimide, 2-(1-(3-dimethylaminopropyl)-indol-3-yl)-3-(indol-3-yl) maleimide, N-(7-dimethylamino-4-methylcoumarin-3-yl)maleimide (DACM), 11H-Benzo[a]fluorene, Benzo[a]pyrene.

In one embodiment, the hedgehog polypeptide can be derivatized using pyrene maleimide, which can be purchased from Molecular Probes (Eugene, Oreg.), e.g., N-(1-pyrene)maleimide or 1-pyrenemethyl iodoacetate (PMIA ester).

For those embodiments wherein the hydrophobic moiety is a polypeptide, the modified hedgehog polypeptide of this invention can be constructed as a fusion protein, containing the hedgehog polypeptide and the hydrophobic moiety as one contiguous polypeptide chain.

In certain embodiments, the lipophilic moiety is an amphipathic polypeptide, such as magainin, cecropin, attacin, melittin, gramicidin S, alpha-toxin of *Staph. aureus*, alamethicin or a synthetic amphipathic polypeptide. Fusogenic coat proteins from viral particles can also be a convenient source of amphipathic sequences for the subject hedgehog proteins

Moreover, mutagenesis can be used to create modified *hh* polypeptides, e.g., for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*). Such modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition. Modified *hedgehog* polypeptides can also include those with altered post-translational processing relative to a naturally occurring *hedgehog* protein, e.g., altered glycosylation, cholesterolization, prenylation and the like.

In one embodiment, the hedgehog therapeutic is a polypeptide encodable by a nucleotide sequence that hybridizes under stringent conditions to a hedgehog coding sequence represented in one or more of SEQ ID Nos:1-7. Appropriate stringency conditions which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C,

are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50°C to a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C.

As described in the literature, genes for other hedgehog proteins, e.g., from other animals, can be obtained from mRNA or genomic DNA samples using techniques well known in the art. For example, a cDNA encoding a *hedgehog* protein can be obtained by isolating total mRNA from a cell, e.g. a mammalian cell, e.g. a human cell, including embryonic cells. Double stranded cDNAs can then be prepared from the total mRNA, and subsequently inserted into a suitable plasmid or bacteriophage vector using any one of a number of known techniques. The gene encoding a *hedgehog* protein can also be cloned using established polymerase chain reaction techniques.

Preferred nucleic acids encode a *hedgehog* polypeptide comprising an amino acid sequence at least 60% homologous or identical, more preferably 70% homologous or identical, and most preferably 80% homologous or identical with an amino acid sequence selected from the group consisting of SEQ ID Nos:8-14. Nucleic acids which encode polypeptides at least about 90%, more preferably at least about 95%, and most preferably at least about 98-99% homology or identity with an amino acid sequence represented in one of SEQ ID Nos:8-14 are also within the scope of the invention.

In addition to native *hedgehog* proteins, *hedgehog* polypeptides preferred by the present invention are at least 60% homologous or identical, more preferably 70% homologous or identical and most preferably 80% homologous or identical with an amino acid sequence represented by any of SEQ ID Nos:8-14. Polypeptides which are at least 90%, more preferably at least 95%, and most preferably at least about 98-99% homologous or identical with a sequence selected from the group consisting of SEQ ID Nos:8-14 are also within the scope of the invention. The only prerequisite is that the *hedgehog* polypeptide is capable of modulating the growth state of peripheral nerve cells.

The term "recombinant protein" refers to a polypeptide of the present invention which is produced by recombinant DNA techniques, wherein generally, DNA encoding a *hedgehog* polypeptide is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein. Moreover, the phrase "derived from", with respect to a recombinant *hedgehog* gene, is meant to include within the meaning of "recombinant protein" those proteins having an amino acid sequence of a

native *hedgehog* protein, or an amino acid sequence similar thereto which is generated by mutations including substitutions and deletions (including truncation) of a naturally occurring form of the protein.

5 The method of the present invention can also be carried out using variant forms of the naturally occurring *hedgehog* polypeptides, e.g., mutational variants.

As is known in the art, *hedgehog* polypeptides can be produced by standard biological techniques or by chemical synthesis. For example, a host cell transfected with a nucleic acid vector directing expression of a nucleotide sequence encoding the subject polypeptides can be cultured under appropriate conditions to allow expression of the peptide to occur. The polypeptide *hedgehog* may be secreted and isolated from a mixture of cells and medium containing the recombinant *hedgehog* polypeptide. Alternatively, the peptide may be retained cytoplasmically by removing the signal peptide sequence from the recombinant *hedgehog* gene and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. 10 Suitable media for cell culture are well known in the art. The recombinant *hedgehog* polypeptide can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for such peptide. In a preferred embodiment, the recombinant *hedgehog* polypeptide is a fusion protein containing a domain which facilitates its purification, such as an *hedgehog*/GST fusion protein. The host cell may be any prokaryotic or eukaryotic cell. 15 20

Recombinant *hedgehog* genes can be produced by ligating nucleic acid encoding an *hedgehog* protein, or a portion thereof, into a vector suitable for expression in either prokaryotic cells, eukaryotic cells, or both. Expression vectors for production of recombinant forms of the subject *hedgehog* polypeptides include plasmids and other vectors. For instance, suitable vectors for the expression of a *hedgehog* polypeptide include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*. 25 30

A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles useful in the introduction of genetic constructs into *S. cerevisiae* (see, for example, Broach *et al.* (1983) in 35 *Experimental Manipulation of Gene Expression*, ed. M. Inouye Academic Press, p. 83, incorporated by reference herein). These vectors can replicate in *E. coli* due to the

presence of the pBR322 ori, and in *S. cerevisiae* due to the replication determinant of the yeast 2 micron plasmid. In addition, drug resistance markers such as ampicillin can be used. In an illustrative embodiment, an *hedgehog* polypeptide is produced recombinantly utilizing an expression vector generated by sub-cloning the coding
5 sequence of one of the *hedgehog* genes represented in SEQ ID Nos:1-7.

The preferred mammalian expression vectors contain both prokaryotic sequences, to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG,
10 pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papillomavirus (BPV-1), or Epstein-Barr virus
15 (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook,
20 Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989) Chapters 16 and 17.

In some instances, it may be desirable to express the recombinant *hedgehog* polypeptide by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-
25 derived vectors (such as the β -gal containing pBlueBac III).

When it is desirable to express only a portion of an *hedgehog* protein, such as a form lacking a portion of the N-terminus, i.e. a truncation mutant which lacks the signal peptide, it may be necessary to add a start codon (ATG) to the oligonucleotide fragment containing the desired sequence to be expressed. It is well known in the art that
30 methionine at the N-terminal position can be enzymatically cleaved by the use of the enzyme methionine aminopeptidase (MAP). MAP has been cloned from *E. coli* (Ben-Bassat et al. (1987) *J. Bacteriol.* 169:751-757) and *Salmonella typhimurium* and its *in vitro* activity has been demonstrated on recombinant proteins (Miller et al. (1987) *PNAS* 84:2718-1722). Therefore, removal of an N-terminal methionine, if desired, can be
35 achieved either *in vivo* by expressing *hedgehog*-derived polypeptides in a host which

produces MAP (e.g., *E. coli* or CM89 or *S. cerevisiae*), or *in vitro* by use of purified MAP (e.g., procedure of Miller et al., *supra*).

Alternatively, the coding sequences for the polypeptide can be incorporated as a part of a fusion gene including a nucleotide sequence encoding a different polypeptide. It is widely appreciated that fusion proteins can also facilitate the expression of proteins, and accordingly, can be used in the expression of the *hedgehog* polypeptides of the present invention. For example, *hedgehog* polypeptides can be generated as glutathione-S-transferase (GST-fusion) proteins. Such GST-fusion proteins can enable easy purification of the *hedgehog* polypeptide, as for example by the use of glutathione-derivatized matrices (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al. (N.Y.: John Wiley & Sons, 1991)). In another embodiment, a fusion gene coding for a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence, can be used to replace the signal sequence which naturally occurs at the N-terminus of the *hedgehog* protein (e.g. of the pro-form, in order to permit purification of the poly(His)-*hedgehog* protein by affinity chromatography using a Ni²⁺ metal resin. The purification leader sequence can then be subsequently removed by treatment with enterokinase (e.g., see Hochuli et al. (1987) *J. Chromatography* 411:177; and Janknecht et al. *PNAS* 88:8972).

Techniques for making fusion genes are known to those skilled in the art. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al. John Wiley & Sons: 1992).

Hedgehog polypeptides may also be chemically modified to create *hedgehog* derivatives by forming covalent or aggregate conjugates with other chemical moieties, such as glycosyl groups, cholesterol, isoprenoids, lipids, phosphate, acetyl groups and the like. Covalent derivatives of *hedgehog* proteins can be prepared by linking the chemical moieties to functional groups on amino acid sidechains of the protein or at the N-terminus or at the C-terminus of the polypeptide.

For instance, *hedgehog* proteins can be generated to include a moiety, other than sequence naturally associated with the protein, that binds a component of the extracellular matrix and enhances localization of the analog to cell surfaces. For example, sequences derived from the fibronectin "type-III repeat", such as a tetrapeptide
5 sequence R-G-D-S (Pierschbacher et al. (1984) *Nature* 309:30-3; and Kornblihtt et al. (1985) *EMBO* 4:1755-9) can be added to the *hedgehog* polypeptide to support attachment of the chimeric molecule to a cell through binding ECM components (Ruoslahti et al. (1987) *Science* 238:491-497; Pierschbacher et al. (1987) *J. Biol. Chem.* 262:17294-8.; Hynes (1987) *Cell* 48:549-54; and Hynes (1992) *Cell* 69:11-25).

10 In a preferred embodiment, the *hedgehog* polypeptide is isolated from, or is otherwise substantially free of, other cellular proteins, especially other extracellular or cell surface associated proteins which may normally be associated with the *hedgehog* polypeptide, unless provided in the form of fusion protein with the *hedgehog* polypeptide. The term "substantially free of other cellular or extracellular proteins"
15 (also referred to herein as "contaminating proteins") or "substantially pure preparations" or "purified preparations" are defined as encompassing preparations of *hedgehog* polypeptides having less than 20% (by dry weight) contaminating protein, and preferably having less than 5% contaminating protein. By "purified", it is meant that the indicated molecule is present in the substantial absence of other biological
20 macromolecules, such as other proteins. The term "purified" as used herein preferably means at least 80% by dry weight, more preferably in the range of 95-99% by weight, and most preferably at least 99.8% by weight, of biological macromolecules of the same type present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 5000, can be present). The term "pure" as used herein
25 preferably has the same numerical limits as "purified" immediately above.

As described above for recombinant polypeptides, isolated *hedgehog* polypeptides can include all or a portion of the amino acid sequences represented in any of SEQ ID Nos:10-18 or 20, or a homologous sequence thereto. Preferred fragments of the subject *hedgehog* proteins correspond to the N-terminal and C-terminal proteolytic
30 fragments of the mature protein. Bioactive fragments of *hedgehog* polypeptides are described in great detail in PCT publications WO 95/18856 and WO 96/17924.

With respect to bioactive fragments of *hedgehog* polypeptide, preferred *hedgehog* therapeutics include at least 50 (contiguous) amino acid residues of a *hedgehog* polypeptide, more preferably at least 100 (contiguous), and even more preferably at least
35 150 (contiguous) residues.

Another preferred *hedgehog* polypeptide which can be included in the *hedgehog* therapeutic is an N-terminal fragment of the mature protein having a molecular weight of approximately 19 kDa.

5 Preferred human *hedgehog* proteins include N-terminal fragments corresponding approximately to residues 24-197 of SEQ ID No. 15, 28-202 of SEQ ID No. 16, and 23-198 of SEQ ID No. 17. By "corresponding approximately" it is meant that the sequence of interest is at most 20 amino acid residues different in length to the reference sequence, though more preferably at most 5, 10 or 15 amino acid different in length.

10 As described above for recombinant polypeptides, isolated *hedgehog* polypeptides can include all or a portion of the amino acid sequences represented in SEQ ID No:8, SEQ ID No:9, SEQ ID No:10, SEQ ID No:11, SEQ ID No:12, SEQ ID No:13 or SEQ ID No:14, or a homologous sequence thereto. Preferred fragments of the subject *hedgehog* proteins correspond to the N-terminal and C-terminal proteolytic fragments of the mature protein. Bioactive fragments of hedgehog polypeptides are
15 described in great detail in PCT publications WO 95/18856 and WO 96/17924.

Still other preferred *hedgehog* polypeptides includes an amino acid sequence represented by the formula A-B wherein: (i) A represents all or the portion of the amino acid sequence designated by residues 1-168 of SEQ ID No:21; and B represents at least one amino acid residue of the amino acid sequence designated by residues 169-221 of
20 SEQ ID No:21; (ii) A represents all or the portion of the amino acid sequence designated by residues 24-193 of SEQ ID No:15; and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No:15; (iii) A represents all or the portion of the amino acid sequence designated by residues 25-193 of SEQ ID No:13; and B represents at least one amino acid residue of the amino acid
25 sequence designated by residues 194-250 of SEQ ID No:13; (iv) A represents all or the portion of the amino acid sequence designated by residues 23-193 of SEQ ID No:11; and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No:11; (v) A represents all or the portion of the amino acid sequence designated by residues 28-197 of SEQ ID No:12; and B represents at least
30 one amino acid residue of the amino acid sequence designated by residues 198-250 of SEQ ID No:12; (vi) A represents all or the portion of the amino acid sequence designated by residues 29-197 of SEQ ID No:16; and B represents at least one amino acid residue of the amino acid sequence designated by residues 198-250 of SEQ ID No:16; or (vii) A represents all or the portion of the amino acid sequence designated by
35 residues 23-193 of SEQ ID No. 17, and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No. 17. In certain

preferred embodiments, A and B together represent a contiguous polypeptide sequence designated sequence, A represents at least 25, 50, 75, 100, 125 or 150 (contiguous) amino acids of the designated sequence, and B represents at least 5, 10, or 20 (contiguous) amino acid residues of the amino acid sequence designated by
5 corresponding entry in the sequence listing, and A and B together preferably represent a contiguous sequence corresponding to the sequence listing entry. Similar fragments from other *hedgehog* also contemplated, e.g., fragments which correspond to the preferred fragments from the sequence listing entries which are enumerated above. In preferred embodiments, the *hedgehog* polypeptide includes a C-terminal glycine (or
10 other appropriate residue) which is derivatized with a cholesterol.

Isolated peptidyl portions of *hedgehog* proteins can be obtained by screening peptides recombinantly produced from the corresponding fragment of the nucleic acid encoding such peptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc
15 chemistry. For example, a *hedgehog* polypeptide of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragments, or preferably divided into overlapping fragments of a desired length. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl fragments which can function as either agonists or antagonists of a wild-type (e.g.,
20 "authentic") *hedgehog* protein. For example, Román et al. (1994) *Eur J Biochem* 222:65-73 describe the use of competitive-binding assays using short, overlapping synthetic peptides from larger proteins to identify binding domains.

The recombinant *hedgehog* polypeptides of the present invention also include homologs of the authentic *hedgehog* proteins, such as versions of those protein which
25 are resistant to proteolytic cleavage, as for example, due to mutations which alter potential cleavage sequences or which inactivate an enzymatic activity associated with the protein. *Hedgehog* homologs of the present invention also include proteins which have been post-translationally modified in a manner different than the authentic protein. Exemplary derivatives of *hedgehog* proteins include polypeptides which lack N-
30 glycosylation sites (e.g. to produce an unglycosylated protein), which lack sites for cholesterolization, and/or which lack N-terminal and/or C-terminal sequences.

Modification of the structure of the subject *hedgehog* polypeptides can also be for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g.,
35 *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*). Such modified peptides, when designed to retain at least one activity of the naturally-occurring form of the protein, are considered functional equivalents of the *hedgehog* polypeptides

described in more detail herein. Such modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition.

It is well known in the art that one could reasonably expect that certain isolated replacements of amino acids, e.g., replacement of an amino acid residue with another related amino acid (i.e. isosteric and/or isoelectric mutations), can be carried out without major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids can be divided into four families: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) nonpolar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In similar fashion, the amino acid repertoire can be grouped as (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine, (3) aliphatic = glycine, alanine, valine, leucine, isoleucine, serine, threonine, with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic = phenylalanine, tyrosine, tryptophan; (5) amide = asparagine, glutamine; and (6) sulfur-containing = cysteine and methionine. (see, for example, *Biochemistry*, 2nd ed., Ed. by L. Stryer, WH Freeman and Co.: 1981). Whether a change in the amino acid sequence of a peptide results in a functional *hedgehog* homolog (e.g. functional in the sense that it acts to mimic or antagonize the wild-type form) can be readily determined by assessing the ability of the variant peptide to produce a response in cells in a fashion similar to the wild-type protein, or competitively inhibit such a response. Polypeptides in which more than one replacement has taken place can readily be tested in the same manner.

It is specifically contemplated that the methods of the present invention can be carried using homologs of naturally occurring hedgehog proteins. In one embodiment, the invention contemplates using hedgehog polypeptides generated by combinatorial mutagenesis. Such methods, as are known in the art, are convenient for generating both point and truncation mutants, and can be especially useful for identifying potential variant sequences (e.g. homologs) that are functional in binding to a receptor for *hedgehog* proteins. The purpose of screening such combinatorial libraries is to generate, for example, novel *hedgehog* homologs which can act as either agonists or antagonist. To illustrate, *hedgehog* homologs can be engineered by the present method to provide more efficient binding to a cognate receptor, such as *patched*, yet still retain at least a portion of an activity associated with *hedgehog*. Thus, combinatorially-derived homologs can be generated to have an increased potency relative to a naturally occurring

form of the protein. Likewise, *hedgehog* homologs can be generated by the present combinatorial approach to act as antagonists, in that they are able to mimic, for example, binding to other extracellular matrix components (such as receptors), yet not induce any biological response, thereby inhibiting the action of authentic *hedgehog* or *hedgehog* agonists. Moreover, manipulation of certain domains of *hedgehog* by the present method can provide domains more suitable for use in fusion proteins, such as one that incorporates portions of other proteins which are derived from the extracellular matrix and/or which bind extracellular matrix components.

To further illustrate the state of the art of combinatorial mutagenesis, it is noted that the review article of Gallop et al. (1994) *J Med Chem* 37:1233 describes the general state of the art of combinatorial libraries as of the earlier 1990's. In particular, Gallop et al state at page 1239 "[s]creening the analog libraries aids in determining the minimum size of the active sequence and in identifying those residues critical for binding and intolerant of substitution". In addition, the Ladner et al. PCT publication WO90/02809, the Goeddel et al. U.S. Patent 5,223,408, and the Markland et al. PCT publication WO92/15679 illustrate specific techniques which one skilled in the art could utilize to generate libraries of *hedgehog* variants which can be rapidly screened to identify variants/fragments which retained a particular activity of the *hedgehog* polypeptides. These techniques are exemplary of the art and demonstrate that large libraries of related variants/truncants can be generated and assayed to isolate particular variants without undue experimentation. Gustin et al. (1993) *Virology* 193:653, and Bass et al. (1990) *Proteins: Structure, Function and Genetics* 8:309-314 also describe other exemplary techniques from the art which can be adapted as means for generating mutagenic variants of *hedgehog* polypeptides.

Indeed, it is plain from the combinatorial mutagenesis art that large scale mutagenesis of *hedgehog* proteins, without any preconceived ideas of which residues were critical to the biological function, and generate wide arrays of variants having equivalent biological activity. Indeed, it is the ability of combinatorial techniques to screen billions of different variants by high throughout analysis that removes any requirement of *a priori* understanding or knowledge of critical residues.

To illustrate, the amino acid sequences for a population of *hedgehog* homologs or other related proteins are aligned, preferably to promote the highest homology possible. Such a population of variants can include, for example, *hedgehog* homologs from one or more species. Amino acids which appear at each position of the aligned sequences are selected to create a degenerate set of combinatorial sequences. In a preferred embodiment, the variegated library of *hedgehog* variants is generated by

combinatorial mutagenesis at the nucleic acid level, and is encoded by a variegated gene library. For instance, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential *hedgehog* sequences are expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g. for phage display) containing the set of *hedgehog* sequences therein.

As illustrated in PCT publication WO 95/18856, to analyze the sequences of a population of variants, the amino acid sequences of interest can be aligned relative to sequence homology. The presence or absence of amino acids from an aligned sequence of a particular variant is relative to a chosen consensus length of a reference sequence, which can be real or artificial.

In an illustrative embodiment, alignment of exons 1, 2 and a portion of exon 3 encoded sequences (e.g. the N-terminal approximately 221 residues of the mature protein) of each of the *Shh* clones produces a degenerate set of *Shh* polypeptides represented by the general formula:

15 C-G-P-G-R-G-X(1)-G-X(2)-R-R-H-P-K-K-L-T-P-L-A-Y-K-Q-F-I-P-N-
V-A-E-K-T-L-G-A-S-G-R-Y-E-G-K-I-X(3)-R-N-S-E-R-F-K-E-L-T-P-N-
Y-N-P-D-I-I-F-K-D-E-E-N-T-G-A-D-R-L-M-T-Q-R-C-K-D-K-L-N-
X(4)-L-A-I-S-V-M-N-X(5)-W-P-G-V-X(6)-L-R-V-T-E-G-W-D-E-D-G-
H-H-X(7)-E-E-S-L-H-Y-E-G-R-A-V-D-I-T-T-S-D-R-D-X(8)-S-K-Y-G-
20 X(9)-L-X(10)-R-L-A-V-E-A-G-F-D-W-V-Y-Y-E-S-K-A-H-I-H-C-S-V-
K-A-E-N-S-V-A-A-K-S-G-G-C-F-P-G-S-A-X(11)-V-X(12)-L-X(13)-
X(14)-G-G-X(15)-K-X(16)-V-K-D-L-X(17)-P-G-D-X(18)-V-L-A-A-D-
X(19)-X(20)-G-X(21)-L-X(22)-X(23)-S-D-F-X(24)-X(25)-F-X(26)-D-R
(SEQ ID No: 21)

25 wherein each of the degenerate positions "X" can be an amino acid which occurs in that position in one of the human, mouse, chicken or zebrafish *Shh* clones, or, to expand the library, each X can also be selected from amongst amino acid residue which would be conservative substitutions for the amino acids which appear naturally in each of those positions. For instance, Xaa(1) represents Gly, Ala, Val, Leu, Ile, Phe, Tyr or Trp ;
30 Xaa(2) represents Arg, His or Lys; Xaa(3) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(4) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(5) represents Lys, Arg, His, Asn or Gln; Xaa(6) represents Lys, Arg or His; Xaa(7) represents Ser, Thr, Tyr, Trp or Phe; Xaa(8) represents Lys, Arg or His; Xaa(9) represents Met, Cys, Ser or Thr; Xaa(10) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(11) represents Leu, Val, Met, Thr or Ser;
35 Xaa(12) represents His, Phe, Tyr, Ser, Thr, Met or Cys; Xaa(13) represents Gln, Asn, Glu, or Asp; Xaa(14) represents His, Phe, Tyr, Thr, Gln, Asn, Glu or Asp; Xaa(15) represents Gln, Asn, Glu, Asp, Thr, Ser, Met or Cys; Xaa(16) represents Ala, Gly, Cys, Leu, Val or Met; Xaa(17) represents Arg, Lys, Met, Ile, Asn, Asp, Glu, Gln, Ser, Thr or

Cys; Xaa(18) represents Arg, Lys, Met or Ile; Xaa(19) represents Ala, Gly, Cys, Asp, Glu, Gln, Asn, Ser, Thr or Met; Xaa(20) represents Ala, Gly, Cys, Asp, Asn, Glu or Gln; Xaa(21) represents Arg, Lys, Met, Ile, Asn, Asp, Glu or Gln; Xaa(22) represent Leu, Val, Met or Ile; Xaa(23) represents Phe, Tyr, Thr, His or Trp; Xaa(24) represents Ile, Val, Leu or Met; Xaa(25) represents Met, Cys, Ile, Leu, Val, Thr or Ser; Xaa(26) represents Leu, Val, Met, Thr or Ser. In an even more expansive library, each X can be selected from any amino acid.

In similar fashion, alignment of each of the human, mouse, chicken and zebrafish *hedgehog* clones, can provide a degenerate polypeptide sequence represented by the general formula:

C-G-P-G-R-G-X(1)-X(2)-X(3)-R-R-X(4)-X(5)-X(6)-P-K-X(7)-L-X(8)-P-L-X(9)-Y-K-Q-F-X(10)-P-X(11)-X(12)-X(13)-E-X(14)-T-L-G-A-S-G-X(15)-X(16)-E-G-X(17)-X(18)-X(19)-R-X(20)-S-E-R-F-X(21)-X(22)-L-T-P-N-Y-N-P-D-I-I-F-K-D-E-E-N-X(23)-G-A-D-R-L-M-T-X(24)-R-C-K-X(25)-X(26)-X(27)-N-X(28)-L-A-I-S-V-M-N-X(29)-W-P-G-V-X(30)-L-R-V-T-E-G-X(31)-D-E-D-G-H-H-X(32)-X(33)-X(34)-S-L-H-Y-E-G-R-A-X(35)-D-I-T-T-S-D-R-D-X(36)-X(37)-K-Y-G-X(38)-L-X(39)-R-L-A-V-E-A-G-F-D-W-V-Y-Y-E-S-X(40)-X(41)-H-X(42)-H-X(43)-S-V-K-X(44)-X(45) (SEQ IDNo:22)

wherein, as above, each of the degenerate positions "X" can be an amino acid which occurs in a corresponding position in one of the wild-type clones, and may also include amino acid residue which would be conservative substitutions, or each X can be any amino acid residue. In an exemplary embodiment, Xaa(1) represents Gly, Ala, Val, Leu, Ile, Pro, Phe or Tyr; Xaa(2) represents Gly, Ala, Val, Leu or Ile; Xaa(3) represents Gly, Ala, Val, Leu, Ile, Lys, His or Arg; Xaa(4) represents Lys, Arg or His; Xaa(5) represents Phe, Trp, Tyr or an amino acid gap; Xaa(6) represents Gly, Ala, Val, Leu, Ile or an amino acid gap; Xaa(7) represents Asn, Gln, His, Arg or Lys; Xaa(8) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(9) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(10) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(11) represents Ser, Thr, Gln or Asn; Xaa(12) represents Met, Cys, Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(13) represents Gly, Ala, Val, Leu, Ile or Pro; Xaa(14) represents Arg, His or Lys; Xaa(15) represents Gly, Ala, Val, Leu, Ile, Pro, Arg, His or Lys; Xaa(16) represents Gly, Ala, Val, Leu, Ile, Phe or Tyr; Xaa(17) represents Arg, His or Lys; Xaa(18) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(19) represents Thr or Ser; Xaa(20) represents Gly, Ala, Val, Leu, Ile, Asn or Gln; Xaa(21) represents Arg, His or Lys; Xaa(22) represents Asp or Glu; Xaa(23) represents Ser or Thr; Xaa(24) represents Glu, Asp, Gln or Asn; Xaa(25) represents Glu or Asp; Xaa(26) represents Arg, His or Lys; Xaa(27) represents Gly, Ala, Val, Leu or Ile; Xaa(28) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; Xaa(29)

represents Met, Cys, Gln, Asn, Arg, Lys or His; Xaa(30) represents Arg, His or Lys; Xaa(31) represents Trp, Phe, Tyr, Arg, His or Lys; Xaa(32) represents Gly, Ala, Val, Leu, Ile, Ser, Thr, Tyr or Phe; Xaa(33) represents Gln, Asn, Asp or Glu; Xaa(34) represents Asp or Glu; Xaa(35) represents Gly, Ala, Val, Leu, or Ile; Xaa(36) represents Arg, His or Lys; Xaa(37) represents Asn, Gln, Thr or Ser; Xaa(38) represents Gly, Ala, Val, Leu, Ile, Ser, Thr, Met or Cys; Xaa(39) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; Xaa(40) represents Arg, His or Lys; Xaa(41) represents Asn, Gln, Gly, Ala, Val, Leu or Ile; Xaa(42) represents Gly, Ala, Val, Leu or Ile; Xaa(43) represents Gly, Ala, Val, Leu, Ile, Ser, Thr or Cys; Xaa(44) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; and Xaa(45) represents Asp or Glu.

There are many ways by which the library of potential *hedgehog* homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential *hedgehog* sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) *Tetrahedron* 39:3; Itakura et al. (1981) *Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules*, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al. (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al. (1984) *Science* 198:1056; Ike et al. (1983) *Nucleic Acid Res.* 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) *Science* 249:386-390; Roberts et al. (1992) *PNAS* 89:2429-2433; Devlin et al. (1990) *Science* 249: 404-406; Cwirla et al. (1990) *PNAS* 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815).

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations, and for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of *hedgehog* homologs. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the illustrative assays described below are amenable to high through-put

analysis as necessary to screen large numbers of degenerate *hedgehog* sequences created by combinatorial mutagenesis techniques.

In one embodiment, the combinatorial library is designed to be secreted (e.g. the polypeptides of the library all include a signal sequence but no transmembrane or cytoplasmic domains), and is used to transfect a eukaryotic cell that can be co-cultured with peripehral nerve cells. A functional *hedgehog* protein secreted by the cells expressing the combinatorial library will diffuse to neighboring peripheral nerve cells and induce a particular biological response, such as proliferation or differentiation. The pattern of detection of such a change in phenotype will resemble a gradient function, and will allow the isolation (generally after several repetitive rounds of selection) of cells producing *hedgehog* homologs active as neurotrophic agents. Likewise, *hedgehog* antagonists can be selected in similar fashion by the ability of the cell producing a functional antagonist to protect neighboring cells (e.g., to inhibit proliferation) from the effect of wild-type *hedgehog* added to the culture media.

To illustrate, target peripheral nerve cells are cultured in 24-well microtitre plates. Other eukaryotic cells are transfected with the combinatorial *hedgehog* gene library and cultured in cell culture inserts (e.g. Collaborative Biomedical Products, Catalog #40446) that are able to fit into the wells of the microtitre plate. The cell culture inserts are placed in the wells such that recombinant *hedgehog* homologs secreted by the cells in the insert can diffuse through the porous bottom of the insert and contact the target cells in the microtitre plate wells. After a period of time sufficient for functional forms of a *hedgehog* protein to produce a measurable response in the target cells, such as growth state, the inserts are removed and the effect of the variant *hedgehog* proteins on the target cells determined. Cells from the inserts corresponding to wells which score positive for activity can be split and re-cultured on several inserts, the process being repeated until the active clones are identified.

In yet another screening assay, the candidate *hedgehog* gene products are displayed on the surface of a cell or viral particle, and the ability of particular cells or viral particles to associate with a *hedgehog*-binding moiety (such as the *patched* protein or other *hedgehog* receptor) via this gene product is detected in a "panning assay". Such panning steps can be carried out on cells cultured from embryos. For instance, the gene library can be cloned into the gene for a surface membrane protein of a bacterial cell, and the resulting fusion protein detected by panning (Ladner et al., WO 88/06630; Fuchs et al. (1991) *Bio/Technology* 9:1370-1371; and Goward et al. (1992) *TIBS* 18:136-140). In a similar fashion, fluorescently labeled molecules which bind *hedgehog* can be used to score for potentially functional *hedgehog* homologs. Cells can be visually inspected and

separated under a fluorescence microscope, or, where the morphology of the cell permits, separated by a fluorescence-activated cell sorter.

In an alternate embodiment, the gene library is expressed as a fusion protein on the surface of a viral particle. For instance, in the filamentous phage system, foreign peptide sequences can be expressed on the surface of infectious phage, thereby conferring two significant benefits. First, since these phage can be applied to affinity matrices at very high concentrations, large number of phage can be screened at one time. Second, since each infectious phage displays the combinatorial gene product on its surface, if a particular phage is recovered from an affinity matrix in low yield, the phage can be amplified by another round of infection. The group of almost identical *E. coli* filamentous phages M13, fd, and f1 are most often used in phage display libraries, as either of the phage gIII or gVIII coat proteins can be used to generate fusion proteins without disrupting the ultimate packaging of the viral particle (Ladner et al. PCT publication WO 90/02909; Garrard et al., PCT publication WO 92/09690; Marks et al. (1992) J. Biol. Chem. 267:16007-16010; Griffiths et al. (1993) EMBO J 12:725-734; Clackson et al. (1991) Nature 352:624-628; and Barbas et al. (1992) PNAS 89:4457-4461).

In an illustrative embodiment, the recombinant phage antibody system (RPAS, Pharmacia Catalog number 27-9400-01) can be easily modified for use in expressing and screening *hedgehog* combinatorial libraries. For instance, the pCANTAB 5 phagemid of the RPAS kit contains the gene which encodes the phage gIII coat protein. The *hedgehog* combinatorial gene library can be cloned into the phagemid adjacent to the gIII signal sequence such that it will be expressed as a gIII fusion protein. After ligation, the phagemid is used to transform competent *E. coli* TG1 cells. Transformed cells are subsequently infected with M13KO7 helper phage to rescue the phagemid and its candidate *hedgehog* gene insert. The resulting recombinant phage contain phagemid DNA encoding a specific candidate *hedgehog*, and display one or more copies of the corresponding fusion coat protein. The phage-displayed candidate *hedgehog* proteins which are capable of binding an *hedgehog* receptor are selected or enriched by panning. For instance, the phage library can be applied to cells which express the *patched* protein and unbound phage washed away from the cells. The bound phage is then isolated, and if the recombinant phage express at least one copy of the wild type gIII coat protein, they will retain their ability to infect *E. coli*. Thus, successive rounds of reinfection of *E. coli*, and panning will greatly enrich for *hedgehog* homologs, which can then be screened for further biological activities in order to differentiate agonists and antagonists.

Combinatorial mutagenesis has a potential to generate very large libraries of mutant proteins, e.g., in the order of 10^{26} molecules. Combinatorial libraries of this size may be technically challenging to screen even with high throughput screening assays such as phage display. To overcome this problem, a new technique has been developed recently, recursive ensemble mutagenesis (REM), which allows one to avoid the very high proportion of non-functional proteins in a random library and simply enhances the frequency of functional proteins, thus decreasing the complexity required to achieve a useful sampling of sequence space. REM is an algorithm which enhances the frequency of functional mutants in a library when an appropriate selection or screening method is employed (Arkin and Yourvan, 1992, *PNAS USA* 89:7811-7815; Yourvan et al., 1992, *Parallel Problem Solving from Nature*, 2., In Maenner and Manderick, eds., Elsevir Publishing Co., Amsterdam, pp. 401-410; Delgrave et al., 1993, *Protein Engineering* 6(3):327-331).

The invention also provides for reduction of the *hedgehog* protein to generate mimetics, e.g. peptide or non-peptide agents, which are able to disrupt binding of a *hedgehog* polypeptide of the present invention with an *hedgehog* receptor. Thus, such mutagenic techniques as described above are also useful to map the determinants of the *hedgehog* proteins which participate in protein-protein interactions involved in, for example, binding of the subject *hedgehog* polypeptide to other extracellular matrix components. To illustrate, the critical residues of a subject *hedgehog* polypeptide which are involved in molecular recognition of an *hedgehog* receptor such as *patched* can be determined and used to generate *hedgehog*-derived peptidomimetics which competitively inhibit binding of the authentic *hedgehog* protein with that moiety. By employing, for example, scanning mutagenesis to map the amino acid residues of each of the subject *hedgehog* proteins which are involved in binding other extracellular proteins, peptidomimetic compounds can be generated which mimic those residues of the *hedgehog* protein which facilitate the interaction. Such mimetics may then be used to interfere with the normal function of a *hedgehog* protein. For instance, non-hydrolyzable peptide analogs of such residues can be generated using benzodiazepine (e.g., see Freidinger et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gama lactam rings (Garvey et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al. (1986) *J Med Chem* 29:295; and Ewenson et al. in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), β -turn dipeptide cores (Nagai et

al. (1985) *Tetrahedron Lett* 26:647; and Sato et al. (1986) *J Chem Soc Perkin Trans* 1:1231), and β -aminoalcohols (Gordon et al. (1985) *Biochem Biophys Res Commun* 126:419; and Dann et al. (1986) *Biochem Biophys Res Commun* 134:71).

Recombinantly produced forms of the hedgehog proteins can be produced using, e.g., expression vectors containing a nucleic acid encoding a *hedgehog* polypeptide, operably linked to at least one transcriptional regulatory sequence. Operably linked is intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence. Regulatory sequences are art-recognized and are selected to direct expression of a *hedgehog* polypeptide. Accordingly, the term transcriptional regulatory sequence includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences, sequences that control the expression of a DNA sequence when operatively linked to it, may be used in these vectors to express DNA sequences encoding *hedgehog* polypeptide. Such useful expression control sequences, include, for example, a viral LTR, such as the LTR of the Moloney murine leukemia virus, the early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage λ , the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α -mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered.

In addition to providing a ready source of hedgehog polypeptides for purification, the gene constructs of the present invention can also be used as a part of a gene therapy protocol to deliver nucleic acids encoding either an agonistic or antagonistic form of a *hedgehog* polypeptide. Thus, another aspect of the invention features expression vectors for *in vivo* transfection of a *hedgehog* polypeptide in

particular cell types so as cause ectopic expression of a *hedgehog* polypeptide in an periperal neurons or other cells associated therewith.

Formulations of such expression constructs may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively
5 delivering the recombinant gene to cells *in vivo*. Approaches include insertion of the hedgehog coding sequence in viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized
10 (e.g. antibody conjugated), polylysine conjugates, gramicidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or CaPO₄ precipitation carried out *in vivo*. It will be appreciated that because transduction of appropriate target cells represents the critical first step in gene therapy, choice of the particular gene delivery system will depend on such factors as the
15 phenotype of the intended target and the route of administration, e.g. locally or systemically. Furthermore, it will be recognized that the particular gene construct provided for *in vivo* transduction of *hedgehog* expression are also useful for *in vitro* transduction of cells, such as for use in the *ex vivo* tissue culture systems described below.

20 A preferred approach for *in vivo* introduction of nucleic acid into a cell is by use of a viral vector containing nucleic acid, e.g. a cDNA, encoding the particular form of the *hedgehog* polypeptide desired. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in
25 the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid.

Retrovirus vectors and adeno-associated virus vectors are generally understood to be the recombinant gene delivery system of choice for the transfer of exogenous genes *in vivo*, particularly into humans. These vectors provide efficient delivery of genes into
30 cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. A major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of
35 retroviruses for gene therapy, and defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood*

76:271). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (*gag*, *pol*, *env*) has been replaced by nucleic acid encoding a hedgehog polypeptide and renders the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include Crip, Cre, 2 and Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including neuronal cells, *in vitro* and/or *in vivo* (see for example Eglitis, et al. (1985) *Science* 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury et al. (1991) *Science* 254:1802-1805; van Beusechem et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Kay et al. (1992) *Human Gene Therapy* 3:641-647; Dai et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu et al. (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

Furthermore, it has been shown that it is possible to limit the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the viral particle (see, for example PCT publications WO93/25234 and WO94/06920). For instance, strategies for the modification of the infection spectrum of retroviral vectors include: coupling antibodies specific for cell surface antigens to the viral *env* protein (Roux et al. (1989) *PNAS* 86:9079-9083; Julan et al. (1992) *J. Gen Virol* 73:3251-3255; and Goud et al. (1983) *Virology* 163:251-254); or coupling cell surface receptor ligands to the viral *env* proteins (Neda et al. (1991) *J Biol Chem* 266:14143-14146). Coupling can be in the form of the chemical cross-linking with a protein or other variety (e.g. lactose to convert the *env* protein to an asialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain antibody/*env* fusion proteins). This technique, while useful to limit or otherwise direct the infection to certain tissue types, can also be used to convert an ecotropic vector in to an amphotropic vector.

Moreover, use of retroviral gene delivery can be further enhanced by the use of tissue- or cell-specific transcriptional regulatory sequences which control expression of the *hedgehog* gene of the retroviral vector.

Another viral gene delivery system useful in the present method utilizes
5 adenovirus-derived vectors. The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner et al. (1988) *BioTechniques* 6:616; Rosenfeld et al. (1991) *Science* 252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the
10 adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses can be advantageous in certain circumstances in that they can be used to infect a wide variety of cell types, including peripheral nerve cells. Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so
15 as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral
20 genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al. cited *supra*; Haj-Ahmand and Graham (1986) *J. Virol.* 57:267). Most replication-defective adenoviral vectors currently in use and therefore favored by the present invention are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material (see, e.g., Jones et al. (1979) *Cell*
25 16:683; Berkner et al., *supra*; and Graham et al. in Methods in Molecular Biology, E.J. Murray, Ed. (Humana, Clifton, NJ, 1991) vol. 7. pp. 109-127). Expression of the inserted *hedgehog* gene can be under control of, for example, the E1A promoter, the major late promoter (MLP) and associated leader sequences, the E3 promoter, or exogenously added promoter sequences.

30 In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a *hedgehog* polypeptide in the tissue of an animal. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral gene delivery systems of the present invention rely
35 on endocytic pathways for the uptake of the *hedgehog* polypeptide gene by the targeted

cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

5 In clinical settings, the gene delivery systems for the therapeutic *hedgehog* gene can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g. Chen et al. (1994) *PNAS* 91: 3054-3057). A *hedgehog* expression construct can be delivered in a gene therapy construct to dermal cells by, e.g., electroporation using techniques described, for example, by Dev et al. ((1994) *Cancer Treat Rev* 20:105-115).

20 The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

25 In yet another embodiment, the *hedgehog* or *ptc* therapeutic can be a "gene activation" construct which, by homologous recombination with a genomic DNA, alters the transcriptional regulatory sequences of an endogenous gene. For instance, the gene activation construct can replace the endogenous promoter of a *hedgehog* gene with a heterologous promoter, e.g., one which causes constitutive expression of the *hedgehog* gene or which causes inducible expression of the gene under conditions different from the normal expression pattern of the gene. Other genes in the *patched* signaling pathway can be similarly targeted. A variety of different formats for the gene activation constructs are available. See, for example, the Transkaryotic Therapies, Inc PCT publications WO93/09222, WO95/31560, WO96/29411, WO95/31560 and WO94/12650.

35 In preferred embodiments, the nucleotide sequence used as the gene activation construct can be comprised of (1) DNA from some portion of the endogenous *hedgehog* gene (exon sequence, intron sequence, promoter sequences, etc.) which direct recombination and (2) heterologous transcriptional regulatory sequence(s) which is to be

operably linked to the coding sequence for the genomic *hedgehog* gene upon recombination of the gene activation construct. For use in generating cultures of *hedgehog* producing cells, the construct may further include a reporter gene to detect the presence of the knockout construct in the cell.

5 The gene activation construct is inserted into a cell, and integrates with the genomic DNA of the cell in such a position so as to provide the heterologous regulatory sequences in operative association with the native *hedgehog* gene. Such insertion occurs by homologous recombination, i.e., recombination regions of the activation construct that are homologous to the endogenous *hedgehog* gene sequence hybridize to the
10 genomic DNA and recombine with the genomic sequences so that the construct is incorporated into the corresponding position of the genomic DNA.

 The terms "recombination region" or "targeting sequence" refer to a segment (i.e., a portion) of a gene activation construct having a sequence that is substantially identical to or substantially complementary to a genomic gene sequence, e.g., including
15 5' flanking sequences of the genomic gene, and can facilitate homologous recombination between the genomic sequence and the targeting transgene construct.

 As used herein, the term "replacement region" refers to a portion of a activation construct which becomes integrated into an endogenous chromosomal location following homologous recombination between a recombination region and a genomic
20 sequence.

 The heterologous regulatory sequences, e.g., which are provided in the replacement region, can include one or more of a variety elements, including: promoters (such as constitutive or inducible promoters), enhancers, negative regulatory elements, locus control regions, transcription factor binding sites, or combinations thereof.

25 Promoters/enhancers which may be used to control the expression of the targeted gene *in vivo* include, but are not limited to, the cytomegalovirus (CMV) promoter/enhancer (Karasuyama et al., 1989, *J. Exp. Med.*, 169:13), the human β -actin promoter (Gunning et al. (1987) *PNAS* 84:4831-4835), the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV
30 LTR) (Klessig et al. (1984) *Mol. Cell Biol.* 4:1354-1362), the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR) (Weiss et al. (1985) *RNA Tumor Viruses*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), the SV40 early or late region promoter (Bernoist et al. (1981) *Nature* 290:304-310; Templeton et al. (1984) *Mol. Cell Biol.*, 4:817; and Sprague et al. (1983) *J. Virol.*,
35 45:773), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (RSV) (Yamamoto et al., 1980, *Cell*, 22:787-797), the herpes simplex virus (HSV)

thymidine kinase promoter/enhancer (Wagner et al. (1981) *PNAS* 82:3567-71), and the herpes simplex virus LAT promoter (Wolfe et al. (1992) *Nature Genetics*, 1:379-384).

In an exemplary embodiment, portions of the 5' flanking region of the human Shh gene are amplified using primers which add restriction sites, to generate the following fragments

5' -

gcgcgccttcgaaGCGAGGCAGCCAGCGAGGGAGAGAGCGAGCGGGCGAGCCGGAGC-
GAGGAAatcgatgcgcgc (primer 1)

10 5' -

gcgcgcagatctGGGAAAGCGCAAGAGAGAGCGCACACGCACACACCCGCCGCGCG-
CACTCGg gatccgcgcgc (primer 2)

As illustrated, primer 1 includes a 5' non-coding region of the human Shh gene and is flanked by an *Asu*II and *Cla*I restriction sites. Primer 2 includes a portion of the 5' non-coding region immediately 3' to that present in primer 1. The hedgehog gene sequence is flanked by *Xho*II and *Bam*HI restriction sites. The purified amplimers are cut with each of the enzymes as appropriate.

The vector pCDNA1.1 (Invitrogen) includes a CMV promoter. The plasmid is cut with with *Asu*II, which cleaves just 3' to the CMV promoter sequence. The *Asu*II/*Cla*I fragment of primer 1 is ligated to the *Asu*II cleavage site of the pcDNA vector. The *Cla*I/*Asu*II ligation destroys the *Asu*II site at the 3' end of a properly inserted primer 1.

The vector is then cut with *Bam*HI, and an *Xho*II/*Bam*HI fragment of primer 2 is ligated to the *Bam*HI cleavage site. As above, the *Bam*HI/*Xho*II ligation destroys the *Bam*HI site at the 5' end of a properly inserted primer 2.

Individual colonies are selected, cut with *Asu*II and *Bam*HI, and the size of the *Asu*II/*Bam*HI fragment determined. Colonies in which both the primer 1 and primer 2 sequences are correctly inserted are further amplified, an cut with *Asu*II and *Bam*HI to produce the gene activation construct

cgaagcgagggcagccagcgagggagagagcgagcgggagcgagccggagcgaggaaATCGA
AGGTTCGAATCCTTCCCCCACCACCATCACTTTCAAAAGTCCGAAAGAATCTGCTCCCT
GCTTGTGTGTTGGAGGTCGCTGAGTAGTGCGCGAGTAAAATTTAAGCTACAACAAGGCA
AGGCTTGACCGACAATTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTC

GCGATGTACGGGCCAGATATACGCGTTGACATTGATTATTGACTAGTTATTAATAGTAA
TCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTAC
GGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTTGACGTCAATAATGA
CGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGACTAT
5 TTACGGTAAACTGCCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCC
TATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTAT
GGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATG
CGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAG
TCTCCACCCCATTTGACGTCAATGGGAGTTTGT TTTTGGCACCAAAATCAACGGGACTTTC
10 CAAAATGTCGTAACAACCTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGG
GAGGTCTATATAAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTAT
CGAAATTAATACGACTCACTATAGGGAGACCCAAGCTTGGTACCGAGCTCGGATCgatc
cgggaaagcgcaagagagagcgcacacgcacacacccgccgcgcgcactcgg

15 In this construct, the flanking primer 1 and primer 2 sequences provide the
recombination region which permits the insertion of the CMV promoter in front of the
coding sequence for the human *Shh* gene. Other heterologous promoters (or other
transcriptional regulatory sequences) can be inserted in a genomic *hedgehog* gene by a
similar method.

20 In still other embodiments, the replacement region merely deletes a negative
transcriptional control element of the native gene, e.g., to activate expression, or ablates
a positive control element, e.g., to inhibit expression of the targeted gene.

V. Exemplary ptc therapeutic compounds.

25 In another embodiment, the subject method is carried out using a ptc therapeutic
composition. Such compositions can be generated with, for example, compounds which
bind to patched and alter its signal transduction activity, compounds which alter the
binding and/or enzymatic activity of a protein (e.g., intracellular) involved in patched
signal pathway, and compounds which alter the level of expression of a hedgehog
protein, a patched protein or a protein involved in the intracellular signal transduction
30 pathway of patched.

The availability of purified and recombinant *hedgehog* polypeptides facilitates
the generation of assay systems which can be used to screen for drugs, such as small
organic molecules, which are either agonists or antagonists of the normal cellular
function of a *hedgehog* and/or patched protein, particularly their role in the pathogenesis
35 of peripheral nerve proliferation and/or differentiation. In one embodiment, the assay

evaluates the ability of a compound to modulate binding between a *hedgehog* polypeptide and a *hedgehog* receptor such as *patched*. In other embodiments, the assay merely scores for the ability of a test compound to alter the signal transduction activity of the *patched* protein. In this manner, a variety of *hedgehog* and/or *ptc* therapeutics, both
5 proliferative and anti-proliferative in activity, can be identified. A variety of assay formats will suffice and, in light of the present disclosure, will be comprehended by skilled artisan.

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of
10 compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the
15 test compound can be generally ignored in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with receptor proteins.

Accordingly, in an exemplary screening assay for *ptc* therapeutics, the compound of interest is contacted with a mixture including a *hedgehog* receptor protein (e.g., a cell
20 expressing the *patched* receptor) and a *hedgehog* protein under conditions in which it is ordinarily capable of binding the *hedgehog* protein. To the mixture is then added a composition containing a test compound. Detection and quantification of receptor/*hedgehog* complexes provides a means for determining the test compound's efficacy at inhibiting (or potentiating) complex formation between the receptor protein
25 and the *hedgehog* polypeptide. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the test compound. Moreover, a control assay can also be performed to provide a baseline for comparison. In the control assay, isolated and purified *hedgehog* polypeptide is added to the receptor protein, and the formation of receptor/*hedgehog* complex is
30 quantitated in the absence of the test compound.

In other embodiments, a *ptc* therapeutic of the present invention is one which disrupts the association of *patched* with *smoothed*.

Agonist and antagonists of peripheral nerve maintenance can be distinguished, and the efficacy of the compound can be assessed, by subsequent testing with peripheral
35 nerve cells, e.g., in culture.

In an illustrative embodiment, the polypeptide utilized as a *hedgehog* receptor can be generated from the *patched* protein. Accordingly, an exemplary screening assay includes all or a suitable portion of the *patched* protein which can be obtained from, for example, the human *patched* gene (GenBank U43148) or other vertebrate sources (see
5 GenBank Accession numbers U40074 for chicken *patched* and U46155 for mouse *patched*), as well as from drosophila (GenBank Accession number M28999) or other invertebrate sources. The *patched* protein can be provided in the screening assay as a whole protein (preferably expressed on the surface of a cell), or alternatively as a fragment of the full length protein which binds to *hedgehog* polypeptides, e.g., as one or
10 both of the substantial extracellular domains (e.g. corresponding to residues Asn120-Ser438 and/or Arg770-Trp1027 of the human *patched* protein - which are also potential antagonists of *hedgehog*-dependent signal transduction). For instance, the *patched* protein can be provided in soluble form, as for example a preparation of one of the extracellular domains, or a preparation of both of the extracellular domains which are
15 covalently connected by an unstructured linker (see, for example, Huston et al. (1988) PNAS 85:4879; and U.S. Patent No. 5,091,513). In other embodiments, the protein can be provided as part of a liposomal preparation or expressed on the surface of a cell. The *patched* protein can derived from a recombinant gene, e.g., being ectopically expressed in a heterologous cell. For instance, the protein can be expressed on oocytes,
20 mammalian cells (e.g., COS, CHO, 3T3 or the like), or yeast cell by standard recombinant DNA techniques. These recombinant cells can be used for receptor binding, signal transduction or gene expression assays. Marigo et al. (1996) *Development* 122:1225-1233 illustrates a binding assay of human *hedgehog* to chick *patched* protein ectopically expressed in *Xenopus laevis* oocytes. The assay system of
25 Marigo et al. can be adapted to the present drug screening assays. As illustrated in that reference, *Shh* binds to the *patched* protein in a selective, saturable, dose-dependent manner, thus demonstrating that *patched* is a receptor for *Shh*.

Complex formation between the *hedgehog* polypeptide and a *hedgehog* receptor may be detected by a variety of techniques. For instance, modulation of the formation of
30 complexes can be quantitated using, for example, detectably labelled proteins such as radiolabelled, fluorescently labelled, or enzymatically labelled *hedgehog* polypeptides, by immunoassay, or by chromatographic detection.

Typically, for cell-free assays, it will be desirable to immobilize either the *hedgehog* receptor or the *hedgehog* polypeptide to facilitate separation of
35 receptor/*hedgehog* complexes from uncomplexed forms of one of the proteins, as well as to accommodate automation of the assay. In one embodiment, a fusion protein can be

provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase/receptor (GST/receptor) fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the *hedgehog* polypeptide, e.g. an ³⁵S-labeled *hedgehog* polypeptide, and the test compound and incubated under conditions conducive to complex formation, e.g. at physiological conditions for salt and pH, though slightly more stringent conditions may be desired. Following incubation, the beads are washed to remove any unbound *hedgehog* polypeptide, and the matrix bead-bound radiolabel determined directly (e.g. beads placed in scintillant), or in the supernatant after the receptor/*hedgehog* complexes are dissociated. Alternatively, the complexes can be dissociated from the bead, separated by SDS-PAGE gel, and the level of *hedgehog* polypeptide found in the bead fraction quantitated from the gel using standard electrophoretic techniques.

Other techniques for immobilizing proteins on matrices are also available for use in the subject assay. For instance, soluble portions of the *hedgehog* receptor protein can be immobilized utilizing conjugation of biotin and streptavidin. For instance, biotinylated receptor molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with the *hedgehog* receptor but which do not interfere with *hedgehog* binding can be derivatized to the wells of the plate, and the receptor trapped in the wells by antibody conjugation. As above, preparations of a *hedgehog* polypeptide and a test compound are incubated in the receptor-presenting wells of the plate, and the amount of receptor/*hedgehog* complex trapped in the well can be quantitated. Exemplary methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the *hedgehog* polypeptide, or which are reactive with the receptor protein and compete for binding with the *hedgehog* polypeptide; as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the *hedgehog* polypeptide. In the instance of the latter, the enzyme can be chemically conjugated or provided as a fusion protein with the *hedgehog* polypeptide. To illustrate, the *hedgehog* polypeptide can be chemically cross-linked or genetically fused with alkaline phosphatase, and the amount of *hedgehog* polypeptide trapped in the complex can be assessed with a chromogenic substrate of the enzyme, e.g. paranitrophenylphosphate. Likewise, a fusion protein comprising the *hedgehog* polypeptide and glutathione-S-transferase can be provided, and complex

formation quantitated by detecting the GST activity using 1-chloro-2,4-dinitrobenzene (Habig et al (1974) *J Biol Chem* 249:7130).

For processes which rely on immunodetection for quantitating one of the proteins trapped in the complex, antibodies against the protein, such as the anti-*hedgehog* antibodies described herein, can be used. Alternatively, the protein to be detected in the complex can be "epitope tagged" in the form of a fusion protein which includes, in addition to the *hedgehog* polypeptide or *hedgehog* receptor sequence, a second polypeptide for which antibodies are readily available (e.g. from commercial sources). For instance, the GST fusion proteins described above can also be used for quantification of binding using antibodies against the GST moiety. Other useful epitope tags include myc-epitopes (e.g., see Ellison et al. (1991) *J Biol Chem* 266:21150-21157) which includes a 10-residue sequence from c-myc, as well as the pFLAG system (International Biotechnologies, Inc.) or the pEZZ-protein A system (Pharmacia, NJ).

Where the desired portion of the *hedgehog* receptor (or other *hedgehog* binding molecule) cannot be provided in soluble form, liposomal vesicles can be used to provide manipulatable and isolatable sources of the receptor. For example, both authentic and recombinant forms of the *patched* protein can be reconstituted in artificial lipid vesicles (e.g. phosphatidylcholine liposomes) or in cell membrane-derived vesicles (see, for example, Bear et al. (1992) *Cell* 68:809-818; Newton et al. (1983) *Biochemistry* 22:6110-6117; and Reber et al. (1987) *J Biol Chem* 262:11369-11374).

In addition to cell-free assays, such as described above, the readily available source of *hedgehog* proteins provided by the art also facilitates the generation of cell-based assays for identifying small molecule agonists/antagonists and the like. Analogous to the cell-based assays described above for screening combinatorial libraries, cells which are sensitive to *hedgehog* induction, e.g. *patched*-expressing cells or other myoblast-derived cells sensitive to *hedgehog* induction, can be contacted with a *hedgehog* protein and a test agent of interest, with the assay scoring for anything from simple binding to the cell to modulation in *hedgehog* inductive responses by the target cell in the presence and absence of the test agent. As with the cell-free assays, agents which produce a statistically significant change in *hedgehog* activities (either inhibition or potentiation) can be identified.

In other embodiments, the cell-based assay scores for agents which disrupt association of *patched* and *smoothed* proteins, e.g., in the cell surface membrane or liposomal preparation.

In addition to characterizing cells that naturally express the *patched* protein, cells which have been genetically engineered to ectopically express *patched* can be utilized for drug screening assays. As an example, cells which either express low levels or lack expression of the *patched* protein, e.g. *Xenopus laevis* oocytes, COS cells or yeast cells, can be genetically modified using standard techniques to ectopically express the *patched* protein. (see Marigo et al., *supra*).

The resulting recombinant cells, e.g., which express a functional *patched* receptor, can be utilized in receptor binding assays to identify agonist or antagonists of *hedgehog* binding. Binding assays can be performed using whole cells. Furthermore, the recombinant cells of the present invention can be engineered to include other heterologous genes encoding proteins involved in *hedgehog*-dependent signal pathways. For example, the gene products of one or more of *smoothed*, *costal-2* and/or *fused* can be co-expressed with *patched* in the reagent cell, with assays being sensitive to the functional reconstitution of the *hedgehog* signal transduction cascade.

Alternatively, liposomal preparations using reconstituted *patched* protein can be utilized. *Patched* protein purified from detergent extracts from both authentic and recombinant origins can be reconstituted in artificial lipid vesicles (e.g. phosphatidylcholine liposomes) or in cell membrane-derived vesicles (see, for example, Bear et al. (1992) *Cell* 68:809-818; Newton et al. (1983) *Biochemistry* 22:6110-6117; and Reber et al. (1987) *J Biol Chem* 262:11369-11374). The lamellar structure and size of the resulting liposomes can be characterized using electron microscopy. External orientation of the *patched* protein in the reconstituted membranes can be demonstrated, for example, by immunoelectron microscopy. The *hedgehog* protein binding activity of liposomes containing *patched* and liposomes without the protein in the presence of candidate agents can be compared in order to identify potential modulators of the *hedgehog-patched* interaction.

The *hedgehog* protein used in these cell-based assays can be provided as a purified source (natural or recombinant in origin), or in the form of cells/tissue which express the protein and which are co-cultured with the target cells. As in the cell-free assays, where simple binding (rather than induction) is the *hedgehog* activity scored for in the assay, the protein can be labelled by any of the above-mentioned techniques, e.g., fluorescently, enzymatically or radioactively, or detected by immunoassay.

In addition to binding studies, functional assays can be used to identify modulators, i.e., agonists or antagonists, of *hedgehog* or *patched* activities. By detecting changes in intracellular signals, such as alterations in second messengers or gene

expression, in *patched*-expressing cells contacted with a test agent, candidate agonists and antagonists to *patched* signaling can be identified.

A number of gene products have been implicated in *patched*-mediated signal transduction, including *patched*, the transcription factor *cubitus interruptus* (ci), the
5 serine/threonine kinase *fused* (fu) and the gene products of *costal-2*, *smoothened* and *suppressor of fused*.

The interaction of a hedgehog protein with *patched* sets in motion a cascade involving the activation and inhibition of downstream effectors, the ultimate consequence of which is, in some instances, a detectable change in the transcription or
10 translation of a gene. Potential transcriptional targets of *patched* signaling are the *patched* gene itself (Hidalgo and Ingham, 1990 *Development* 110, 291-301; Marigo et al., 1996) and the vertebrate homologs of the drosophila cubitus interruptus gene, the *GLI* genes (Hui et al. (1994) *Dev Biol* 162:402-413). *Patched* gene expression has been shown to be induced in cells of the limb bud and the neural plate that are responsive to
15 *Shh*. (Marigo et al. (1996) *PNAS*, in press; Marigo et al. (1996) *Development* 122:1225-1233). The *GLI* genes encode putative transcription factors having zinc finger DNA binding domains (Orenic et al. (1990) *Genes & Dev* 4:1053-1067; Kinzler et al. (1990) *Mol Cell Biol* 10:634-642). Transcription of the *GLI* gene has been reported to be upregulated in response to *hedgehog* in limb buds, while transcription of the *GLI3* gene
20 is downregulated in response to *hedgehog* induction (Marigo et al. (1996) *Development* 122:1225-1233). By selecting transcriptional regulatory sequences from such target genes, e.g. from *patched* or *GLI* genes, that are responsible for the up- or down regulation of these genes in response to *patched* signalling, and operatively linking such promoters to a reporter gene, one can derive a transcription based assay which is
25 sensitive to the ability of a specific test compound to modify *patched* signalling pathways. Expression of the reporter gene, thus, provides a valuable screening tool for the development of compounds that act as agonists or antagonists of *ptc* induction of differentiation/quiescence.

Reporter gene based assays of this invention measure the end stage of the above
30 described cascade of events, e.g., transcriptional modulation. Accordingly, in practicing one embodiment of the assay, a reporter gene construct is inserted into the reagent cell in order to generate a detection signal dependent on *ptc* signaling. To identify potential regulatory elements responsive to *ptc* signaling present in the transcriptional regulatory sequence of a target gene, nested deletions of genomic clones of the target gene can be
35 constructed using standard techniques. See, for example, Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989);

U.S. Patent 5,266,488; Sato et al. (1995) *J Biol Chem* 270:10314-10322; and Kube et al. (1995) *Cytokine* 7:1-7. A nested set of DNA fragments from the gene's 5'-flanking region are placed upstream of a reporter gene, such as the luciferase gene, and assayed for their ability to direct reporter gene expression in *patched* expressing cells. Host cells
5 transiently transfected with reporter gene constructs can be scored for the induction of expression of the reporter gene in the presence and absence of *hedgehog* to determine regulatory sequences which are responsive to *patched*-dependent signalling.

In practicing one embodiment of the assay, a reporter gene construct is inserted into the reagent cell in order to generate a detection signal dependent on second
10 messengers generated by induction with *hedgehog* protein. Typically, the reporter gene construct will include a reporter gene in operative linkage with one or more transcriptional regulatory elements responsive to the *hedgehog* activity, with the level of expression of the reporter gene providing the *hedgehog*-dependent detection signal. The amount of transcription from the reporter gene may be measured using any method
15 known to those of skill in the art to be suitable. For example, mRNA expression from the reporter gene may be detected using RNase protection or RNA-based PCR, or the protein product of the reporter gene may be identified by a characteristic stain or an intrinsic activity. The amount of expression from the reporter gene is then compared to the amount of expression in either the same cell in the absence of the test compound (or
20 *hedgehog*) or it may be compared with the amount of transcription in a substantially identical cell that lacks the target receptor protein. Any statistically or otherwise significant difference in the amount of transcription indicates that the test compound has in some manner altered the signal transduction of the *patched* protein, e.g., the test compound is a potential ptc therapeutic.

As described in further detail below, in preferred embodiments the gene product
25 of the reporter is detected by an intrinsic activity associated with that product. For instance, the reporter gene may encode a gene product that, by enzymatic activity, gives rise to a detection signal based on color, fluorescence, or luminescence. In other preferred embodiments, the reporter or marker gene provides a selective growth
30 advantage, e.g., the reporter gene may enhance cell viability, relieve a cell nutritional requirement, and/or provide resistance to a drug.

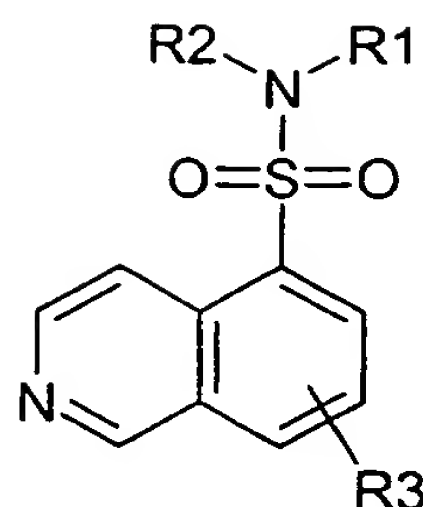
Preferred reporter genes are those that are readily detectable. The reporter gene may also be included in the construct in the form of a fusion gene with a gene that includes desired transcriptional regulatory sequences or exhibits other desirable
35 properties. Examples of reporter genes include, but are not limited to CAT (chloramphenicol acetyl transferase) (Alton and Vapnek (1979), *Nature* 282: 864-869)

luciferase, and other enzyme detection systems, such as beta-galactosidase; firefly luciferase (deWet et al. (1987), Mol. Cell. Biol. 7:725-737); bacterial luciferase (Engebrecht and Silverman (1984), PNAS 1: 4154-4158; Baldwin et al. (1984), Biochemistry 23: 3663-3667); alkaline phosphatase (Toh et al. (1989) Eur. J. Biochem. 182: 231-238, Hall et al. (1983) J. Mol. Appl. Gen. 2: 101), human placental secreted alkaline phosphatase (Cullen and Malim (1992) Methods in Enzymol. 216:362-368).

Transcriptional control elements which may be included in a reporter gene construct include, but are not limited to, promoters, enhancers, and repressor and activator binding sites. Suitable transcriptional regulatory elements may be derived from the transcriptional regulatory regions of genes whose expression is induced after modulation of a *patched* signal transduction pathway. The characteristics of preferred genes from which the transcriptional control elements are derived include, but are not limited to, low or undetectable expression in quiescent cells, rapid induction at the transcriptional level within minutes of extracellular stimulation, induction that is transient and independent of new protein synthesis, subsequent shut-off of transcription requires new protein synthesis, and mRNAs transcribed from these genes have a short half-life. It is not necessary for all of these properties to be present.

In yet other embodiments, second messenger generation can be measured directly in the detection step, such as mobilization of intracellular calcium, phospholipid metabolism or adenylate cyclase activity are quantitated, for instance, the products of phospholipid hydrolysis IP₃, DAG or cAMP could be measured. For example, recent studies have implicated protein kinase A (PKA) as a possible component of *hedgehog/patched* signaling (Hammerschmidt et al. (1996) *Genes & Dev* 10:647). High PKA activity has been shown to antagonize *hedgehog* signaling in these systems. Although it is unclear whether PKA acts directly downstream or in parallel with *hedgehog* signaling, it is possible that *hedgehog* signalling occurs via inhibition of PKA activity. Thus, detection of PKA activity provides a potential readout for the instant assays.

In a preferred embodiment, the *ptc* therapeutic is a PKA inhibitor. A variety of PKA inhibitors are known in the art, including both peptidyl and organic compounds. For instance, the *ptc* therapeutic can be a 5-isoquinolinesulfonamide, such as represented in the general formula:



wherein,

R_1 and R_2 each can independently represent hydrogen, and as valence and stability permit a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-R_8$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$ -lower alkyl, $-(CH_2)_m-O$ -lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$ -lower alkyl, $-(CH_2)_m-S$ -lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$, or

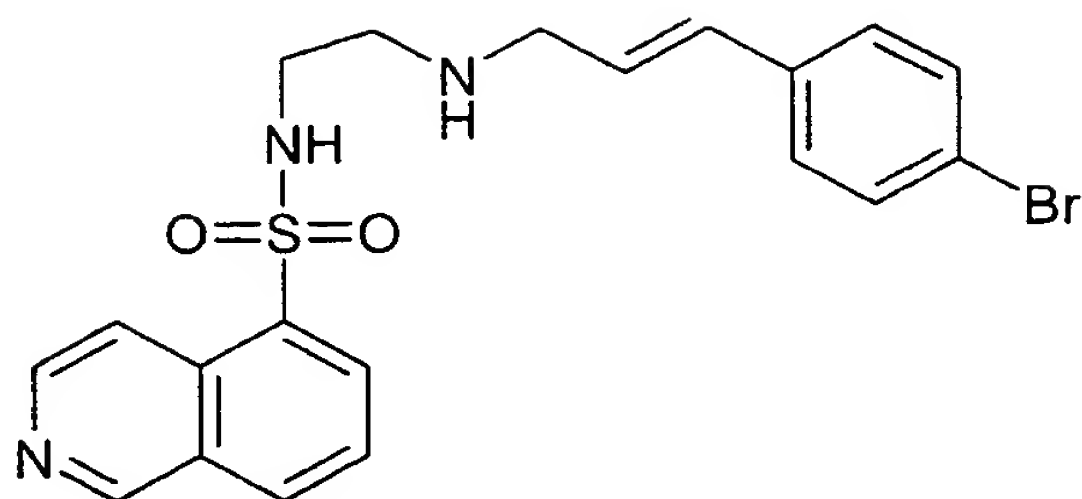
R_1 and R_2 taken together with N form a heterocycle (substituted or unsubstituted);

R_3 is absent or represents one or more substitutions to the isoquinoline ring such as a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-R_8$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$ -lower alkyl, $-(CH_2)_m-O$ -lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$ -lower alkyl, $-(CH_2)_m-S$ -lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$;

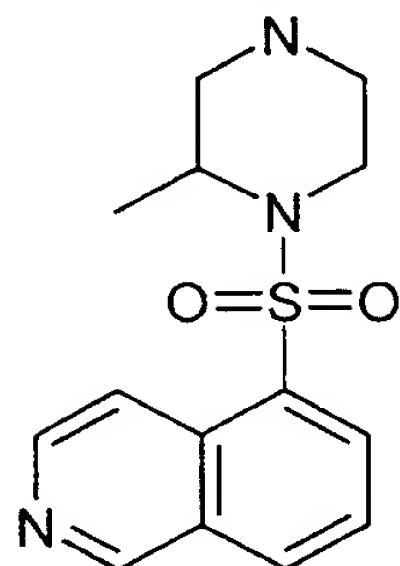
R_8 represents a substituted or unsubstituted aryl, aralkyl, cycloalkyl, cycloalkenyl, or heterocycle; and

n and m are independently for each occurrence zero or an integer in the range of 1 to 6.

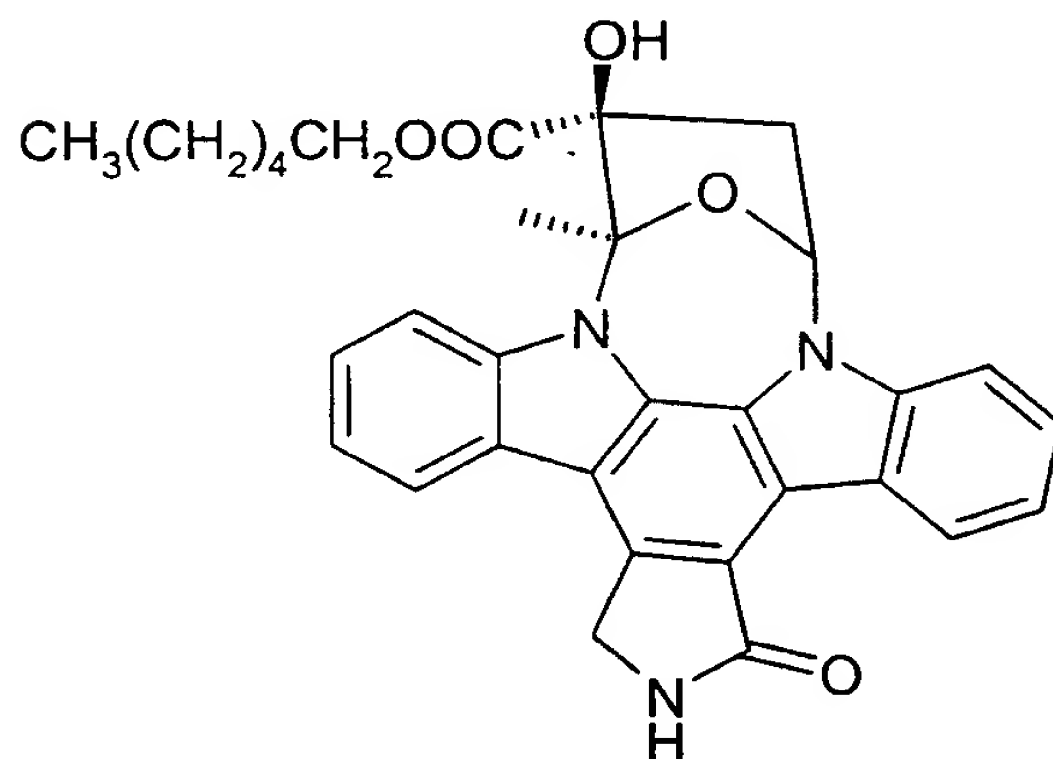
In a preferred embodiment, the PKA inhibitor is N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H-89; Calbiochem Cat. No. 371963), e.g., having the formula:



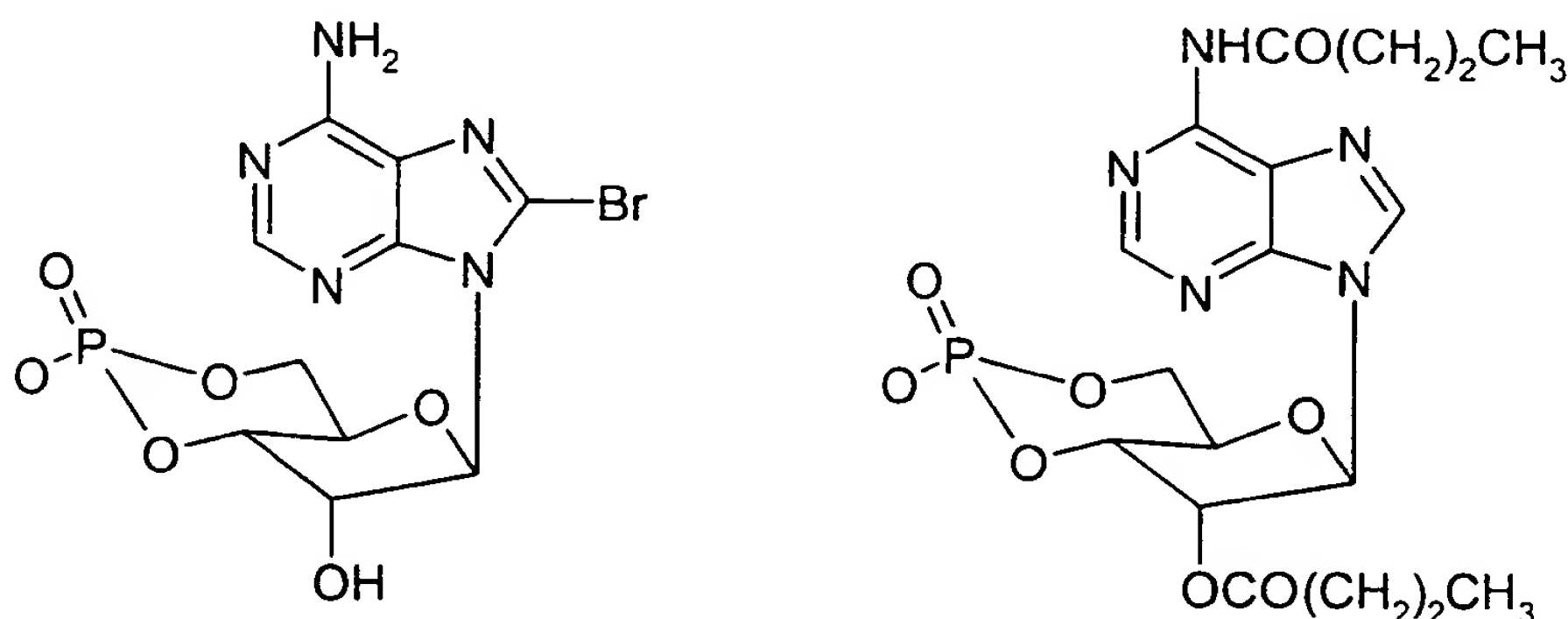
In another embodiment, the PKA inhibitor is 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7; Calbiochem Cat. No. 371955), e.g., having the formula:



- 5 In still other embodiments, the PKA inhibitor is KT5720 (Calbiochem Cat. No. 420315),
having the structure



A variety of nucleoside analogs are also useful as PKA inhibitors. For example, the subject method can be carried out cyclic AMP analogs which inhibit the kinase activity of PKA, as for example, 8-bromo-cAMP or dibutyryl-cAMP



Exemplary peptidyl inhibitors of PKA activity include the PKA Heat Stable Inhibitor (isoform α ; see, for example, Calbiochem Cat. No. 539488, and Wen et al. (1995) *J Biol Chem* 270:2041).

- 5 Certain *hedgehog* receptors may stimulate the activity of phospholipases. Inositol lipids can be extracted and analyzed using standard lipid extraction techniques. Water soluble derivatives of all three inositol lipids (IP₁, IP₂, IP₃) can also be quantitated using radiolabelling techniques or HPLC.

- 10 The mobilization of intracellular calcium or the influx of calcium from outside the cell may be a response to *hedgehog* stimulation or lack thereof. Calcium flux in the reagent cell can be measured using standard techniques. The choice of the appropriate calcium indicator, fluorescent, bioluminescent, metallochromic, or Ca⁺⁺-sensitive microelectrodes depends on the cell type and the magnitude and time constant of the event under study (Borle (1990) *Environ Health Perspect* 84:45-56). As an exemplary method of Ca⁺⁺ detection, cells could be loaded with the Ca⁺⁺-sensitive fluorescent dye fura-2 or indo-1, using standard methods, and any change in Ca⁺⁺ measured using a fluorometer.

- 15 In certain embodiments of the assay, it may be desirable to screen for changes in cellular phosphorylation. As an example, the drosophila gene *fused* (fu) which encodes a serine/threonine kinase has been identified as a potential downstream target in *hedgehog* signaling. (Preat et al., 1990 *Nature* 347, 87-89; Therond et al. 1993, *Mech. Dev.* 44, 65-80). The ability of compounds to modulate serine/threonine kinase activation could be screened using colony immunoblotting (Lyons and Nelson (1984) *Proc. Natl. Acad. Sci. USA* 81:7426-7430) using antibodies against phosphorylated serine or threonine residues. Reagents for performing such assays are commercially available, for example, phosphoserine and phosphothreonine specific antibodies which measure increases in phosphorylation of those residues can be purchased from commercial sources.

In yet another embodiment, the *ptc* therapeutic is an antisense molecule which inhibits expression of a protein involved in a *patched*-mediated signal transduction pathway. To illustrate, by inhibiting the expression of a protein which are involved in *patched* signals, such as fused, costal-2, smoothened and/or Gli genes, the ability of the
5 patched signal pathway(s) to inhibit proliferation of a cell can be altered, e.g., potentiated or repressed.

As used herein, "antisense" therapy refers to administration or *in situ* generation of oligonucleotide probes or their derivatives which specifically hybridize (e.g. bind) under cellular conditions with cellular mRNA and/or genomic DNA encoding a
10 *hedgehog* protein, *patched*, or a protein involved in *patched*-mediated signal transduction. The hybridization should inhibit expression of that protein, e.g. by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, "antisense"
15 therapy refers to the range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

An antisense construct of the present invention can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the target cellular mRNA. Alternatively,
20 the antisense construct is an oligonucleotide probe which is generated *ex vivo* and which, when introduced into the cell causes inhibition of expression by hybridizing with the mRNA and/or genomic sequences of a target gene. Such oligonucleotide probes are preferably modified oligonucleotide which are resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases, and is therefore stable *in vivo*. Exemplary nucleic
25 acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van der Krol et al. (1988) *Biotechniques* 6:958-976; and Stein et al. (1988) *Cancer Res* 48:2659-2668.

30 Several considerations should be taken into account when constructing antisense oligonucleotides for the use in the methods of the invention: (1) oligos should have a GC content of 50% or more; (2) avoid sequences with stretches of 3 or more G's; and (3) oligonucleotides should not be longer than 25-26 mers. When testing an antisense oligonucleotide, a mismatched control can be constructed. The controls can be
35 generated by reversing the sequence order of the corresponding antisense oligonucleotide in order to conserve the same ratio of bases.

In an illustrative embodiment, the *ptc* therapeutic can be an antisense construct for inhibiting the expression of *patched*, e.g., to mimic the inhibition of *patched* by *hedgehog*. Exemplary antisense constructs include:

5'-GTCCTGGCGCCGCGCCGCGCGTCGCC

5 5'-TTCCGATGACCGGCCTTTCGCGGTGA

5'-GTGCACGGAAAGGTGCAGGCCACACT

VI. Exemplary pharmaceutical preparations of *hedgehog* and *ptc* therapeutics

The source of the *hedgehog* and *ptc* therapeutics to be formulated will depend on the particular form of the agent. Small organic molecules and peptidyl fragments can be chemically synthesized and provided in a pure form suitable for pharmaceutical/cosmetic usage. Products of natural extracts can be purified according to techniques known in the art. For example, the Cox et al. U.S. Patent 5,286,654 describes a method for purifying naturally occurring forms of a secreted protein and can be adapted for purification of *hedgehog* polypeptides. Recombinant sources of *hedgehog* polypeptides are also available. For example, the gene encoding *hedgehog* polypeptides, are known, *inter alia*, from PCT publications WO 95/18856 and WO 96/17924.

Those of skill in treating peripheral neuropathies can determine the effective amount of an *hedgehog* or *ptc* therapeutic to be formulated in a pharmaceutical or cosmetic preparation.

The *hedgehog* or *ptc* therapeutic formulations used in the method of the invention are most preferably applied in the form of appropriate compositions. As appropriate compositions there may be cited all compositions usually employed for systemically or topically administering drugs. The pharmaceutically acceptable carrier should be substantially inert, so as not to act with the active component. Suitable inert carriers include water, alcohol polyethylene glycol, mineral oil or petroleum gel, propylene glycol and the like.

To prepare the pharmaceutical compositions of this invention, an effective amount of the particular *hedgehog* or *ptc* therapeutic as the active ingredient is combined in intimate admixture with a pharmaceutically acceptable carrier, which carrier may take a wide variety of forms depending on the form of preparation desired for administration. These pharmaceutical compositions are desirable in unitary dosage form suitable, particularly, for administration orally, rectally, percutaneously, or by parenteral

injection. For example, in preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed such as, for example, water, glycols, oils, alcohols and the like in the case of oral liquid preparations such as suspensions, syrups, elixirs and solutions; or solid carriers such as starches, sugars, kaolin, lubricants, binders, disintegrating agents and the like in the case of powders, pills, capsules, and tablets. Because of their ease in administration, tablets and capsules represents the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. For parenteral compositions, the carrier will usually comprise sterile water, at least in large part, though other ingredients, for example, to aid solubility, may be included. Injectable solutions, for example, may be prepared in which the carrier comprises saline solution, glucose solution or a mixture of saline and glucose solution. Injectable suspensions may also be prepared in which case appropriate liquid carriers, suspending agents and the like may be employed. Also included are solid form preparations which are intended to be converted, shortly before use, to liquid form preparations. In the compositions suitable for percutaneous administration, the carrier optionally comprises a penetration enhancing agent and/or a suitable wetting agent, optionally combined with suitable additives of any nature in minor proportions, which additives do not introduce a significant deleterious effect on the skin.

In addition to the direct topical application of the preparations they can be topically administered by other methods, for example, encapsulated in a temperature and/or pressure sensitive matrix or in film or solid carrier which is soluble in body fluids and the like for subsequent release, preferably sustained-release of the active component.

As appropriate compositions for topical application there may be cited all compositions usually employed for topically administering therapeutics, e.g., creams, gellies, dressings, shampoos, tinctures, pastes, ointments, salves, powders, liquid or semiliquid formulation and the like. Application of said compositions may be by aerosol e.g. with a propellant such as nitrogen carbon dioxide, a freon, or without a propellant such as a pump spray, drops, lotions, or a semisolid such as a thickened composition which can be applied by a swab. In particular compositions, semisolid compositions such as salves, creams, pastes, gellies, ointments and the like will conveniently be used.

It is especially advantageous to formulate the subject compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used in the specification and claims herein refers to physically discrete units suitable as unitary dosages, each unit containing a predetermined quantity of active ingredient calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. Examples of such dosage unit forms are tablets (including scored or coated

tablets), capsules, pills, powders packets, wafers, injectable solutions or suspensions, teaspoonfuls, tablespoonfuls and the like, and segregated multiples thereof.

The pharmaceutical preparations of the present invention can be used, as stated above, for the many applications which can be considered cosmetic uses. Cosmetic compositions known in the art, preferably hypoallergic and pH controlled are especially preferred, and include toilet waters, packs, lotions, skin milks or milky lotions. The preparations contain, besides the hedgehog or ptc therapeutic, components usually employed in such preparations. Examples of such components are oils, fats, waxes, surfactants, humectants, thickening agents, antioxidants, viscosity stabilizers, chelating agents, buffers, preservatives, perfumes, dyestuffs, lower alkanols, and the like. If desired, further ingredients may be incorporated in the compositions, e.g. antiinflammatory agents, antibacterials, antifungals, disinfectants, vitamins, sunscreens, antibiotics, or other anti-acne agents.

Examples of oils comprise fats and oils such as olive oil and hydrogenated oils; waxes such as beeswax and lanolin; hydrocarbons such as liquid paraffin, ceresin, and squalane; fatty acids such as stearic acid and oleic acid; alcohols such as cetyl alcohol, stearyl alcohol, lanolin alcohol, and hexadecanol; and esters such as isopropyl myristate, isopropyl palmitate and butyl stearate. As examples of surfactants there may be cited anionic surfactants such as sodium stearate, sodium cetylsulfate, polyoxyethylene laurylether phosphate, sodium N-acyl glutamate; cationic surfactants such as stearyldimethylbenzylammonium chloride and stearyltrimethylammonium chloride; ampholytic surfactants such as alkylaminoethylglycine hydrochloride solutions and lecithin; and nonionic surfactants such as glycerin monostearate, sorbitan monostearate, sucrose fatty acid esters, propylene glycol monostearate, polyoxyethylene oleylether, polyethylene glycol monostearate, polyoxyethylene sorbitan monopalmitate, polyoxyethylene coconut fatty acid monoethanolamide, polyoxypropylene glycol (e.g. the materials sold under the trademark "Pluronic"), polyoxyethylene castor oil, and polyoxyethylene lanolin. Examples of humectants include glycerin, 1,3-butylene glycol, and propylene glycol; examples of lower alcohols include ethanol and isopropanol; examples of thickening agents include xanthan gum, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, polyethylene glycol and sodium carboxymethyl cellulose; examples of antioxidants comprise butylated hydroxytoluene, butylated hydroxyanisole, propyl gallate, citric acid and ethoxyquin; examples of chelating agents include disodium edetate and ethanehydroxy diphosphate; examples of buffers comprise citric acid, sodium citrate, boric acid, borax, and disodium hydrogen phosphate; and

examples of preservatives are methyl parahydroxybenzoate, ethyl parahydroxybenzoate, dehydroacetic acid, salicylic acid and benzoic acid.

For preparing ointments, creams, toilet waters, skin milks, and the like, typically from 0.01 to 10% in particular from 0.1 to 5% and more in particular from 0.2 to 2.5% of the active ingredient, e.g., of the hedgehog or ptc therapeutic, will be incorporated in the compositions. In ointments or creams, the carrier for example consists of 1 to 20%, in particular 5 to 15% of a humectant, 0.1 to 10% in particular from 0.5 to 5% of a thickener and water; or said carrier may consist of 70 to 99%, in particular 20 to 95% of a surfactant, and 0 to 20%, in particular 2.5 to 15% of a fat; or 80 to 99.9% in particular 90 to 99% of a thickener; or 5 to 15% of a surfactant, 2-15% of a humectant, 0 to 80% of an oil, very small ($< 2\%$) amounts of preservative, coloring agent and/or perfume, and water. In a toilet water, the carrier for example consists of 2 to 10% of a lower alcohol, 0.1 to 10% or in particular 0.5 to 1% of a surfactant, 1 to 20%, in particular 3 to 7% of a humectant, 0 to 5% of a buffer, water and small amounts ($< 2\%$) of preservative, dyestuff and/or perfume. In a skin milk, the carrier typically consists of 10-50% of oil, 1 to 10% of surfactant, 50-80% of water and 0 to 3% of preservative and/or perfume. In the aforementioned preparations, all % symbols refer to weight by weight percentage.

Particular compositions for use in the method of the present invention are those wherein the hedgehog or ptc therapeutic is formulated in liposome-containing compositions. Liposomes are artificial vesicles formed by amphiphatic molecules such as polar lipids, for example, phosphatidyl cholines, ethanolamines and serines, sphingomyelins, cardiolipins, plasmalogens, phosphatidic acids and cerebiosides. Liposomes are formed when suitable amphiphathic molecules are allowed to swell in water or aqueous solutions to form liquid crystals usually of multilayer structure comprised of many bilayers separated from each other by aqueous material (also referred to as coarse liposomes). Another type of liposome known to be consisting of a single bilayer encapsulating aqueous material is referred to as a unilamellar vesicle. If water-soluble materials are included in the aqueous phase during the swelling of the lipids they become entrapped in the aqueous layer between the lipid bilayers.

Water-soluble active ingredients such as, for example, various salt forms of a hedgehog polypeptide, are encapsulated in the aqueous spaces between the molecular layers. The lipid soluble active ingredient of hedgehog or ptc therapeutic, such as an organic mimetic, is predominantly incorporated into the lipid layers, although polar head groups may protude from the layer into the aqueous space. The encapsulation of these compounds can be achieved by a number of methods. The method most commonly used involves casting a thin film of phospholipid onto the walls of a flask by evaporation

from an organic solvent. When this film is dispersed in a suitable aqueous medium, multilamellar liposomes are formed. Upon suitable sonication, the coarse liposomes form smaller similarly closed vesicles.

Water-soluble active ingredients are usually incorporated by dispersing the cast
5 film with an aqueous solution of the compound. The unencapsulated compound is then removed by centrifugation, chromatography, dialysis or other art-known suitable procedures. The lipid-soluble active ingredient is usually incorporated by dissolving it in the organic solvent with the phospholipid prior to casting the film. If the solubility of the material in the lipid phase is not exceeded or the amount present is not in excess of that
10 which can be bound to the lipid, liposomes prepared by the above method usually contain most of the material bound in the lipid bilayers; separation of the liposomes from unencapsulated material is not required.

A particularly convenient method for preparing liposome formulated forms of hedgehog and ptc therapeutics is the method described in EP-A-253,619, incorporated
15 herein by reference. In this method, single bilayered liposomes containing encapsulated active ingredients are prepared by dissolving the lipid component in an organic medium, injecting the organic solution of the lipid component under pressure into an aqueous component while simultaneously mixing the organic and aqueous components with a high speed homogenizer or mixing means, whereupon the liposomes are formed
20 spontaneously.

The single bilayered liposomes containing the encapsulated hedgehog or ptc therapeutic can be employed directly or they can be employed in a suitable pharmaceutically acceptable carrier for topical administration. The viscosity of the liposomes can be increased by the addition of one or more suitable thickening agents
25 such as, for example xanthan gum, hydroxypropyl cellulose, hydroxypropyl methylcellulose and mixtures thereof. The aqueous component may consist of water alone or it may contain electrolytes, buffered systems and other ingredients, such as, for example, preservatives. Suitable electrolytes which can be employed include metal salts such as alkali metal and alkaline earth metal salts. The preferred metal salts are calcium
30 chloride, sodium chloride and potassium chloride. The concentration of the electrolyte may vary from zero to 260 mM, preferably from 5 mM to 160 mM. The aqueous component is placed in a suitable vessel which can be adapted to effect homogenization by effecting great turbulence during the injection of the organic component. Homogenization of the two components can be accomplished within the vessel, or,
35 alternatively, the aqueous and organic components may be injected separately into a mixing means which is located outside the vessel. In the latter case, the liposomes are

formed in the mixing means and then transferred to another vessel for collection purpose.

The organic component consists of a suitable non-toxic, pharmaceutically acceptable solvent such as, for example ethanol, glycerol, propylene glycol and polyethylene glycol, and a suitable phospholipid which is soluble in the solvent. Suitable phospholipids which can be employed include lecithin, phosphatidylcholine, phosphatidylserine, phosphatidylethanol-amine, phosphatidylinositol, lysophosphatidylcholine and phosphatidyl glycerol, for example. Other lipophilic additives may be employed in order to selectively modify the characteristics of the liposomes. Examples of such other additives include stearylamine, phosphatidic acid, tocopherol, cholesterol and lanolin extracts.

In addition, other ingredients which can prevent oxidation of the phospholipids may be added to the organic component. Examples of such other ingredients include tocopherol, butylated hydroxyanisole, butylated hydroxytoluene, ascorbyl palmitate and ascorbyl oleate. Preservatives such as benzoic acid, methyl paraben and propyl paraben may also be added.

Apart from the above-described compositions, use may be made of covers, e.g. plasters, bandages, dressings, gauze pads and the like, containing an appropriate amount of a hedgehog or ptc therapeutic. In some cases use may be made of plasters, bandages, dressings, gauze pads and the like which have been impregnated with a topical formulation containing the therapeutic formulation.

Exemplification

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Example 1: Evaluation of the neuroprotective action sonic hedge hog in a cisplatin-induced neuropathy

The use of antiviral or anticancer chemotherapy may induce a severe neuropathy, that implies a reduction of the dosage used and hence a risk of unsuccess of the treatment. For example cisplatin is largely used for the treatment of tumors of the bladder, testis or ovary; however the dosage is limited because of the appearance of a

partially irreversible toxic neuropathy, with a preference for the sensory fibers of large diameter that modifies the proprioceptive sensitivity (Mollman, 1990). However there is presently no real treatment to cure or prevent such neurotoxicity.

It should be noted that NGF has been shown to be able to limit the importance of neuropathies induced by such chemotherapeutic agents (Apfel et al, 1991, Apfel et al, 1992). Two other peptides (NT3 and an ACTH analog) have also been tested in a similar model (Gao et al, 1995; Hamers et al, 1993). sonic hedge hog has been implicated in antero-posterior patterning of the developing chick limb (Riddle et al, 1993) and in motor neurons differentiation (Roelink et al, 1995). The present study was performed in order to measure the effect of Sonic Hedge Hog (SHH) as protective with regard to cisplatin-induced neuropathy. Behavioral and EMG measurements showed that SHH efficiently protected peripheral neurons against neuropathy, particularly at the highest concentration tested (500 ug/kg).

1) Materials and Methods

1.1) Animals housing and treatment

Thirty nine mice were included in this study and divided into 4 groups of 9-10 mice 38-40 g at onset; one group was treated with SHH (50 ug/kg, s.c.) 3 times per week; the second group received a dosage of 500 ug/kg; a third group was a vehicle group. These three groups were also treated with cisplatin (as described below). A fourth group was a control group without cisplatin administration but treated with 500 ug/kg SHH (control 500). Stock solution SHH (2.8mg/ml) was stored frozen at -70°C; on the day of use a vial was diluted to 0.2mg/ml with PBS and protein was mixed gently by peptetting. The animals were housed in plastic cages at room temperature in a 12: 12 h light-dark cycle. The mice had free access to food and water.

Animals were weighted once weekly and checked for their general behavior walking attitude and general outlook. Electromyographical and behavioral tests were also performed once weekly.

1.2) Cisplatin administration

Cisplatin was administered as an aqueous solution (1 mg/ml) at a dosage of 2 mg/kg i.p once daily during 14 consecutive days (cumulative dose). In order to avoid an important loss of weight of the animals, a Ringer-lactate solution was administered daily (0.4 ml/day i.p).

1.3) Behavioral testing

1.3.1) Pain threshold measurement

1.3.1.1) Tail flick test

5 The tail of the mouse was placed under a shutter-controlled lamp as a heat source. The latency before the mouse flicked its tail from the heat was recorded. A sensory alteration would increase the latency to flick.

1.3.1.2) Hot plate test

10 The animal was placed inside a glass cylinder of 17 cm height and 9 cm diameter on a hot plate at 52°C. The animal's behavior was observed, particularly the licking of a foot, the jump in the cylinder and the adjusted leap. The latency before licking its foot or before jumping to escape the heat was recorded. If the thermal sensitivity was altered, the time needed to feel the pain would be increased.

1.3.2) Motor coordination measurement

15 1.3.2.1) Rotarod test

The ability of an animal to stay on a rotating dowel (rotarod) is a good mean to measure the motor coordination and the proprioceptive sensitivity. The apparatus consisted of a rod, 1cm in diameter, which turned at 12 rpm. The mice were tested for their ability to balance on the rotating bar during 180 sec maximum time (Tilson and
20 Mitchell, 1984).

1.3.2.2) Walk on a rod

The animals were placed on a rod 1.5 cm in diameter and 40 cm long, that was situated horizontally at 50 cm over the floor; they were placed at one extremity and tended to reach the other end, that consisted of a wooden platform. The time needed to
25 reach the platform was related to the motor coordination: the longest it was, the most important the motor deficit.

1.3.3) Muscle performance measurement

1.3.3.1) Muscular endurance

30 The muscular strength was evaluated by measuring the ability of an animal to hold a weight of 32 grams when it was lifted by the tail. The animal was allowed to use either two or the four legs. The time during which it held the weight was recorded, with a maximum of 60 sec, and reflected the muscular endurance.

1.3.3.2) Maximal strength

The maximal muscle strength was measured with an isometric transducer attached to a piece of wire. When the animal held the wire with either two or the four legs, it was slowly moved backwards until it released the wire. The transducer measured the maximal strength; results are given in newton.

1.4) Electrophysiological measurement

Sensitive evoked response: Sensory nerve conduction velocity (SNCV).

Animals were anaesthetized with ketamine chlorhydrate (Ketalar) and diazepam (Valium) (1 ml/kg of a solution containing 11.25 mg ketalar and 0.375 mg of valium; i.p). Electrophysiological recordings were performed using a Neuromatic electromyogram (EMG) apparatus (Dantec, Les Ulis, France). Mice were deeply anaesthetized and normal body temperature maintained with a heating lamp.

The sensitive evoked response was measured in the caudal nerve. Stimulation of the caudal nerve was performed at the base of the tail, with two electrodes (one active, one reference) separated by 3mm; a unipolar recording needle was placed in a proximal site at approximately 40 mm. Sensory nerve velocity was recorded according to orthodromic conduction (from the tip of tail to the base). A ground needle electrode was inserted between the stimulating and recording electrode needles. The SNCV was calculated according to the distance between the two active electrodes.

1.5) Statistical studies.

The Electrophysiological and behavioral data were statistically analyzed by an analysis of variance with repeated measures (ANOVA). Following these analysis, a Scheffe's post hoc test was used to check for differences between individual groups.

2) Results

2.1) General survey

General behavior of animals was normal during the initial 2 weeks of study; however locomotor activity decreased while neuropathy was progressing, hair color changed and finally animals were almost immobile in their cages. Weight decreased strikingly after 2 weeks and remained low in vehicle group until 5 weeks. (Fig. 1; difference between treatments significant at $p < 0.0001$; correlation between treatment effect and time changes significant at $p < 0.0001$). However weight of animals treated with SHH (at both concentrations) increased immediately after the end of cisplatin administration and was almost normal at the end of study. In vehicle group, weight only

started to increase at 5 weeks and was significantly below normal value at the end of study.

As a consequence of cisplatin toxicity, some animals died during the study, starting at 3 weeks. However number of surviving animals was higher in SHH treated group, compare to vehicle (Fig. 2). On the other hand, 3 controlSHH animals died during anaesthesia at 1 and 5 weeks.

2.2) EMG: Sensory nerve conduction velocity (SNCV)

According to EMG measurements, the neuropathy was found to appear after 1 week of cisplatin administration, was maximal at 3 weeks (delayed effect) and recover period went up to 8 weeks.

In standard conditions SNCV varied between 47 and 51 m/s for mice of 8 weeks of age. After cisplatin administration, SNCV decreased significantly in vehicle and SHH50 groups (Fig. 3; difference between treatments significant at $p < 0.0001$; correlation between treatment effect and time changes significant at $p < 0.0001$); recovery started immediately after end of cisplatin administration in SHH50 group, but was delayed one week later in vehicle group. Normal SNCV values were recovered after 8 weeks. However no significant decrease was found in SHH500 or control500 groups.

2.3) Behavioral testing

2.3.1) Pain threshold measurement

2.3.1.1) Tail flick test

Latency to flick the tail was increased after cisplatin administration in vehicle group, with a maximum at 4 weeks (Fig. 4; difference between treatments significant at $p < 0.0001$; correlation between treatment effect and time changes significant at $p < 0.0002$). A similar tendency was found in SHH50 group, but the curve was always below vehicle, i.e pain threshold defect was less important. In SHH50 group, latency increase was only transiently measured at 3 weeks.

2.3.1.2) Hot plate test

The latency before licking the paw did not vary much during the study, except a transient increase in vehicle group at 6 weeks (Fig. 5; difference between treatments not significant; correlation between treatment effect and time changes not significant). It

should be noted that a great variation was found at that time and no significant difference was seen.

When pain was more important, mice tried to escape by jumping; the latency before first jump was recorded. It was found to be increased in vehicle group until 7 weeks and in SHH50 until 2 weeks (Fig. 6); the difference between treatments was only statistically significant at 6 weeks because of large variations in vehicle group (time course significant at $p < 0.0001$; correlation between treatment effect and time changes not significant). A minor increase in SHH500 group was also measured until 3 weeks; values returned to normal thereafter and they were significantly lower than vehicle at 5 weeks.

After prolonged exposure to heat, mice escaped by jumping onto the rim of cylinder; some increase of the latency to escape was found at 2 weeks (particularly in SHH50 group) without reaching significance (Fig. 7). A greater increase was transiently found in vehicle group after 5 weeks and difference was statistically significant when compared to SHH treated groups (time course significant at $p < 0.0001$; correlation between treatment effect and time changes significant at $p < 0.0001$).

2.3.2) Motor coordination measurement

2.3.2.1) Rotarod test

The ability of an animal to stay on a rotating rod was found to be significantly decreased in vehicle group, with a minimum performance at 3 weeks (Fig. 8). No decrease was measured in control500 or SHH500 groups and only a transient decrease at 2 weeks in SHH50 group (difference between treatments significant at $p < 0.0001$; correlation between treatment effect and time changes significant at $p < 0.0072$).

2.3.2.2) Walk on a rod

The time needed to walk on the rod in order to reach the platform significantly increased in vehicle group at 2 and 5 weeks, but only at 2 weeks in SHH50 group (Fig. 9; difference between treatments significant at $p < 0.0015$; correlation between treatment effect and time changes significant at $p < 0.0001$). No increase was found in SHH500 group, except at 3 weeks.

2.3.3) Muscle performance measurement

2.3.3.1) Muscular endurance

When mice were allowed to use all 4 limbs to pull the wire, no decrease of muscular endurance was measured, except in vehicle group at 5 weeks (Fig. 10a; difference between treatments not significant; correlation between treatment effect and time changes not significant). When mice were allowed to use only forelimbs to pull the wire, some decrease in muscular endurance was measured in vehicle group, but not in SHH50 or SHH500 groups (Fig. 10b; difference between treatments not significant; correlation between treatment effect and time changes not significant). It should be noted that some decrease was also transiently found in control500 at 4 and 5 weeks.

2.3.3.2) Maximal strength

The maximal muscle strength exerted by the 4 limbs was decreased after 1-2 weeks in all cisplatin-treated groups (Fig. 11a; time course significant at $p < 0.019$; correlation between treatment effect and time changes not significant). Recovery occurred at 5 weeks in SHH50 and SHH500 groups, but only at 7 weeks in vehicle group. No decrease was found in control.

The maximal muscle strength exerted by the forelimbs progressively decreased in vehicle group, with a minimum value at 6 weeks and recovery at 7 weeks (Fig. 11b; difference between treatments significant at $p < 0.014$; correlation between treatment effect and time changes significant at $p < 0.005$). A transient (and not significant decrease) was found in SHH50 at 2 weeks and no decrease was measured in SHH500 or control500 groups.

3) Discussion

The results obtained in the present study show that SHH was able to protect peripheral nerve against neuropathy induced by cisplatin, particularly at the highest concentration. The most striking effect was observed on SNCV, where no decrease was noticed in SHH500 group. In SHH50 group, SNCV decrease similar to vehicle was measured at 2 weeks; however recovery already began at 3 weeks, i.e one week earlier than in vehicle group. Similarly sensory defect is shown with tail flick test in vehicle group that lasted throughout the study while it was only transient in SHH500 (at 3 weeks). Sensory defect measured on the hot plate (first jump) was found until week 5 in vehicle group and week 2 in SHH50. No significant defect was measured in SHH500 group. Proprioceptive defect is also suggested by rotarod data in vehicle group until week 7 and transiently in SHH50 at week 2. No defect was found in SHH500 group. However these changes may also be related to alteration of motor coordination.

Initial sensory neuropathy is known to extend towards motor impairment in patients treated with cisplatin. Similarly in the present study, muscle performance was impaired in the forelimbs endurance test in vehicle group, but not in any SHH group. Maximal muscle strength exerted by the 5 limbs was decreased in vehicle and both SHH groups, but recovery of function occurred earlier in SHH groups. No such decrease was found in the forelimbs maximal strength test in SHH500 group.

Weight variation is a good indicator of general metabolism of the animals. It decreased strikingly at 2 weeks following cisplatin administration and lasted until week 5 in vehicle group; in both SHH groups recovery occurred immediately after the end of cisplatin administration. Similarly animal survival was improved by SHH treatment.

It is concluded that SHH treatment with 500 ug/kg avoids neuropathy impairment in most tests or accelerates recovery when some defect is measured. Treatment with 50 ug/kg does not protect to the same extent, but also improves recovery (SNCV, jump, rotarod, muscle strength). Difference in time course of recovery is 2 weeks or more, when compared to vehicle group. These effects are similar to those observed with NGF or ACTH analog treatment in a similar paradigm (Apfel et al, 1992; Hamers et al, 1993); recovery of weight loss and SNCV decrease were also observed after end of cisplatin treatment. Dosage of ACTH was similar (75 ug/kg s.c every 48h), while amount of NGF was 10 times higher (5 mg/kg 3 times per week) and 1 mg/kg had no effect.

It should be noted that naive animals treated with 500 ug/kg SHH (but without cisplatin) did not show any impairment, except in forelimbs endurance. However as mentioned 3 animals of this group died during anaesthesia, at 1 and 5 weeks. Taken together with the absence of other signs of impairment in this group, it is most improbable that this occurrence may be due to toxicity of prolonged administration of the compound. However a similar study with lower dosage (100 or 200 ug/kg SHH) may be useful.

5) References for Example 1

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Example 2: Evaluation of peripherhal nerves in normal and transgenic Dhh knockout mice.

- 15 We also undertook a comparison of the electrophysiology and morphology of peripheral nerve cells and bundles in normal mice and in transgenic mice in which the Dhh gene has been disrupted (the "Dhh^{-/-}" phenotype).

- 20 Adult mice were anesthetized with 0.5cc of ketamine/xylazine (diluted 1:10 with sterile saline) delivered by i.p. injection. The hair over the hindlimbs was shaved and the legs were taped in an extended position. Their core temperature was maintained at 38°C with an infrared lamp. A pair of surface recording electrodes were placed on the bottom of each foot; one over the intrinsic plantar muscles, the other more distally. The sciatic nerve was stimulated both proximally (at the level of the L5 vertebrae) and distally (the tibial nerve was stimulated at the ankle) with a pair of subcutaneous electrodes using a Dantec Neuromatic 2000. The stimulus strength was gradually increased until a maximal compound muscle action potential was obtained. The distance between the proximal and distal stimulation sites was measured and used to calculate the motor nerve conduction velocity.

- 30 Figure 12 illustrates that motor neuron conductance velocities are diminished in the Dhh^{-/-} mice, e.g., showing a functional deficit in peripheral nerve of Dhh^{-/-} mice.

The morphology of the peripheral nerve bundles in these mice were also observed (compare Figure 13A with 13B, and 14A with 14B). The integrity of the

epineurial and perineurial sheath was altered in the *Dhh*^{-/-} mice. In another line of experiments, we tested the ability of Shh and Dhh to alter the proliferation of perineurial cells. Based on BrdU incorporation, both hedgehog proteins were able to increase proliferation of perineurial cells, but Dhh was dramatically more effective.

5 In addition to suggesting a role for hedgehog gene products in peripheral neuropathies, the observation that hedgehog proteins can induce proliferation of perineurial cells suggests that antagonists of hedgehog activity may be useful in disorders marked by unwanted proliferation of perineurial cells. For instance, localized hypertrophic mononeuropathy (LHM) is a rare focal neuropathy associated with
10 perineurial cell proliferation due to an undefined stimulus. Perineuriomas. Likewise, in leprosy neuropathy, proliferation of perineurial cells can be implicated in the abnormal multilayered appearance of the perineurium. Antagonists of hedgehog signalling may therefor be useful to inhibit proliferation of perineurial cells in the treatment of such disorders.

15

Example 3: Evaluation of the neuroprotective action sonic hedgehog in a taxol-induced neuropathy

The use of antiviral or anticancer chemotherapy may induce a severe neuropathy, that implies reduction of the dosage used and enhances the risk of unsuccess of the
20 treatment. For example; taxol is used for the treatment of ovarian cancer or melanoma ; however the dosage is limited because of the appearance of a sensory toxic neuropathy (Lipton et al. 1989). It should be noted that NGF has been shown to limit the importance of neuropathies induced by such chemotherapeutic agents. The present study was designed to investigate the potency of Shh to protect against taxol-induced neuropathy.
25 As shown in Figures 16 and 17, Shh has positive effects on taxol-treated mice, e.g., enhancing their ability to walk the length of a long suspended rod and to stay on a rotating drum (the so-called rotorod). Both are measures of motor ability and coordination:

30 1) Animals

Sixty four 22-24 g male Swiss mice (IFFA-CREDO, L'Arbresle, France) were used in this study. They were housed in collective cages (4-5 per cage) and maintained in a room with controlled temperature (21-22°C) and light under a reversed 12-12 light-dark cycle (light on at 7 p.m.), with food and water available ad libitum. All
35 experiments were carried out in accordance with institutional guidelines.

2) Pharmacological treatment

Taxol (Sigma, l'Isle d'Abeau, France) was diluted in saline using cremophor 10 % V/v (Sigma) (20 mg taxol, 1 ml cremophor, 9 ml saline), and administered intraperitoneally (IP) as a volume of 10 ml/kg at the dose of 20 mg/kg once daily during 7 consecutive days. Shh was supplied by Biogen (Cambridge, MA, USA). Stock solutions Shh (2 mg/ml and 0.2 mg/ml) were stored at -70°C. Shh and vehicle solutions were labeled as A, B or C in order to perform a double-blind study. On the day of use, vials containing Shh or vehicle (A, B or Q were diluted to 1/40 in saline (200 µl sample + 7.8 ml saline) and injected as a volume of 10 ml/kg. Shh (50 or 500 µg/kg) or saline was administered subcutaneously (SC) 3 times per week (n=16 mice per group). These 3 groups were also treated with taxol. A fourth group consisting of a control group received cremophor IP and saline SC (n = 16). Shh treatment started from the first day of taxol administration on and lasted for 2 weeks.

15

3) Behavioral testing

Sensorimotor tests were performed once a week for 3 weeks. These tests were always done one day before electrophysiological (EPG) recordings. Each group was divided in two subgroups (series 1 and 2). Series 1 was tested on Mondays for behavioral tests and on Tuesdays for EPG test, Series 2 was tested on Wednesdays for behavioral tests and on Thursdays for EPG analysis. Behavioral testing was performed on day 0 (baseline, before taxol intoxication), day 7 (after 6 days of taxol injection), and day 14 (6 days after taxol discontinuation). EPG measurements, were performed before taxol intoxication (day 1), on day 8 (one day after the last injection of taxol), and on day 15 (7 days after discontinuing taxol). The first injection of taxol was performed on day 1, immediately after EPG recording.

3.1) Motor coordination measurements

Walking test: The apparatus used was a rod of 1.5 cm diameter and 80 cm long, maintained horizontally 40 cm above a table. The rod was graduated starting in the middle (0 cm) towards the two ends (40 cm) allowing to measure the distance walked by the animal.

Animals were tested once each week. Three consecutive trials were performed. For each trial (60 s maximum), each mouse was placed in the middle of the rod and the

time needed to walk the 40 cm distance was recorded. Should the animal fall down or be unable to walk the 40 cm distance, 60 s were credited. For each animal, the mean time of the 3 trials was calculated. This time reflects the motor coordination performance.

5 Rotarod test: The ability of an animal to remain on a rotating rod (rotarod) reflects motor coordination and proprioceptive sensitivity. The apparatus used was a 3 cm diameter automated rod (Bloseb, Paris, France) with 12 rotations per min.

Animals were tested once each week. The mouse was placed on the rotating rod, and the time it remained on rod was recorded (300 s maximum). If the animal falls before 300 s, an additional trial is performed (3 trials maximum).

10

3.2) Muscular power

15 Maximal strength: The maximal muscle strength was measured with an isometric dynamometer connected to a grid. Once the animal was holding the grid with either two or the four paws, it was slowly moved backwards until it released it. The dynamometer measured the maximal strength developed ; results are given in N. Two trials per session were performed. The mean of both trials was calculated for each animal.

20 Muscular endurance: The muscular endurance was evaluated by measuring the time (maximum of 60 s) during which an animal, lifted by the tail, was able to hold a weight of 38 g. The animal was allowed to use either two or the four paws. Two consecutive trials were performed. The mean of both trials was calculated.

3.3) Sensitivity tests

25 Tail flick test: The apparatus consisted of a shutter-controlled lamp as a heat source (Bioseb). Each weekly session consisted of two consecutive trials with an interval of about 1 min and the mean was calculated.

Example 4: Evaluation of the neuroprotective action sonic hedge hog on spinal motor neurons

30 Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder primarily involving motoneurons (Ripps and al., 1995). Overexpression of a mutated human superoxide dismutase gene in mice causes a progressive paralytic disease as result of motoneuron loss in lumbar spinal cord (Mohajeri and al., 1998). The

SODI-G93A transgenic mouse model, used for preclinical drug studies in ALS (Gurney, 1997 ; Morrison and al., 1996), is a good model to explore etiological mechanisms and to screen potential therapeutics. The present experiment, the results of which are illustrated in Figures 18-21, demonstrate the positive effects of hedgehog treatment on the survival of spinal motoneurons in SOD transgenic mice, a mouse model of ALS.

With intent to complete a study which analyses the effect of SHH by electromyographical and sensorimotor tests on progressive motoneurons degeneration in transgenic mice overexpressing mutant human superoxide dismutase, nervous tissue was harvested and histological studies performed on lumbar spinal cord sections.

10

1) Animals and treatment

SOD mice were genotyped by polymerized chain reaction (PCR) amplification of DNA extracted from the tail, 30 days after birth. DNA (10 ng) was added to 50 µl of mix reaction containing MgCl₂ and deoxynucleotide triphosphate mixture. The reaction uses primer sequences set b for exon 4 described by Rosen and al. (1993) that hybridize to opposite strands and flank the target DNA sequence that is to be amplified using a GeneAmp PCR 2400 thermal cycler (Perkin-Elmer, USA). The elongation of the primers is catalyzed by Tag DNA Polymerase (Appligene Oncor, France), a heat-stable DNA polymerase. A repetitive series of 30 cycles involving template denaturation (20 seconds at 92°C, primer annealing (20 seconds at 60°C) and extension of the annealed primers (20 seconds at 72°C) by Tag DNA Polymerase results in exponential accumulation of a specific DNA fragment. The resulting PCR products were electrophoresed on an 2% agarose gel and visualized with ethidium bromide (Sigma, L'Isle d'Abeau, France).

Twelve transgenic G93A heterozygotes mice (6 males and 6 females) were included in the study and were divided into 2 groups of 6 mice. One group was treated with vehicle and the other with SHH at 500 µg/kg of body weight. They were housed in plastic cages and had free access to food and water. The local was maintained at a constant temperature of 22°C and humidity of 55% under conventional conditions and on a 12h light / 12h dark photocycle (light on 7 p.m.).

SHH was administered subcutaneously (SC) 3 times per week starting at 60 days of age, until 100 days.

2) Tissue harvesting and staining

Mice at 100 days of age were anaesthetized with 60 mg/kg ketamine hydrochloride (Ketalar) and 2 mg/kg diazepam. (Valium). They were perfused transcardially with phosphate-buffered saline (PBS) containing 0.1% heparin (Sigma, L'Isle d'Abeau, France). Then, animals were perfused with 4% paraformaldehyde in PBS until they became rigid. Spinal cord was harvested and postfixed overnight. Tissue was then placed in 30% sucrose (Sigma, L'Isle d'Abeau, France) at 4°C until use.

Spinal cord was frozen in cold isopentane (Prolabo, Fontenay-sous-bois, France), embedded with Tissue-tek O.C.T. compound (Miles, USA) and sections (thickness: 30 μ m) were made with a cryostat (Leica Jung CM 1800, Rueil-Malmaison, France). The sections were stained with a 0.1% aqueous solution of cresyl violet (Sigma, L'Isle d'Abeau, France) for 30 to 45 seconds and then dehydrated and mounted in Eukitt (O. Kindler GmbH and Co., Freiburg). Only sections from lumbar segment were examined and to avoid the possibility of a given neuron being counted twice in two contiguous sections, only series of one section out of two were collected. Twenty seven to thirty one sections were obtained from a given lumbar segment. Sections were observed using an optical microscope (Nikon, Japan). Results are expressed as the mean number of cells per animal counted in ventral horns on both sides.

3) Statistical analysis

Values are given as mean \pm s.e.mean. Differences between control group and SHH 500 group were evaluated by one factor ANOVA test using Statview Student vl.O VF software.

4) Results

Figure 18 shows that the group treated with SHH at the dose of 500 μ g/kg of body weight displayed a greater number of motoneurons than the control group, but difference was not significant [$F(1,10)=1.3$; N.S.]. It should be noted that in each group, the number of cells counted in the lumbar segment of the spinal cord of 1 mouse was much lower than the others (2Y0 for control group and 1Y0 for SHH 500 group, Table 1) and these mice were from the same littermate. It was therefore suggested to exclude these mice from the analysis. Figure 19 shows that without Y0 littermate, the number of cells counted was significantly different between control group and SHH 500 group, and that s.e.m. was much smaller. The number of cells in SHH 500 group was 15% higher than in the control group [$F(1,8)=13.7$; $p < 0.01$].

Table 1: Number of cells counted in each group (individual values)

		<u>Identification</u>	<u>Sex</u>	<u>Number of cells</u>
5	Control group	2W2.....	Female.....	874
		2Y0.....	Female.....	678
		1Z0.....	Male.....	920
		1Z2.....	Male.....	932
		2Z0.....	Female.....	835
10	SHH 500 group	1A0.....	Male.....	851
		2T20.....	Female.....	1055
		2T3.....	Female.....	928
		2T1.....	Female.....	1007
		1U1.....	Male.....	1111
15		1U2.....	Male.....	985
		1Y0.....	Male.....	589

In order to further analyze data, motoneurons numbers measured in males and females were analyzed separately. Figure 20 shows that in males there was no statistical difference between control group and SHH 500 group [$F(1,4)=0.0014$; N.S.]. However in females, the number of cells counted in SHH 500 group was significantly higher than in the control group [$F(1,4)=8.1$; $p < 0.05$] as shown in figure 21. These data suggest that SHH compound significantly improved motoneurons survival particularly in females mice.

The observation of individual data in control group shows that the number of cells counted in females, even not significantly, was lower than in males (795.7 ± 59.9 vs 901.0 ± 25.2). This difference may be explained by an earlier start of disease in females than in males. It may be interesting to measure the effects of SHH on motoneurons survival at later age and also to check if hormonal treatment may be able to synergies with SHH administration. In addition, it may be important to begin SHH treatment earlier, as data suggest that neuromuscular impairments may already be present at 60 days.

5) References

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Example 5: Evaluating actions of hedgehog proteins on galactose intoxication-mediated neuropathies

10 Galactose intoxication is a mean of inducing neuropathy and disrupting neurotrophic support to peripheral nerve cells in rats. Feeding rats diets high in galactose causes morphologic abnormalities in, e.g., Schwann cells and muscle that are accompanied by a neuropathy characterized by axonal atrophy and slowing nerve conduction velocities.

15 Adapting a methodology set forth in Mizisin et al. (1997) J. Neuropath Exp Neurol 56: 1290-1301, the effects of hedgehog treatment on functional and structural disorders in nerves of galactosemic rats can be assessed.

As illustrated in Figure 23, treatment with Shh can improve nerve conductance in the galactose intoxicated animal.

20 **Example 6: Evaluating the ability of treatment with hedgehog proteins to protect against diabetic neuropathy**

In rats, i.p. injections of streptozotocine (STZ) can be used to generate an animal model of diabetic neuropathy. Utilizing such procedures as described in Garrett et al. (1997) Neurosci. Lett 222:191-194 the ability of hedgehog treatment to protect STZ-induced 25 neuropathies can be assessed.

Example 7: Evaluating the effect of Hedgehog treatment on nerve crush injury

Hedgehog proteins improve functional recovery following sciatic nerve crush injury. Male CD-1 mice (25-30 g) were given a bilateral sciatic nerve crush and monitored daily 30 for functional recovery by assessing their ability to grip a wire mesh with each hindfoot. See Figure 22. The data are expressed the average number of grip failures for the right and left foot in 10 trials. Mice were treated every other day beginning on the day of

nerve crush with either vehicle (control group), pegylated isoleucine-isoleucine sonic hedgehog (Shh-PEG) at a dose of 1 mg/kg s.c. or isoleucine-isoleucine sonic hedgehog murine Ig fusion protein (Shh-Ig) at doses of 1 or 5 mg/kg s.c. The values represent the mean \pm S.E.M. for 14 mice per group. * $P < 0.05$ for all Shh groups compared to vehicle-treated control, Student-Newman-Keuls test.

All of the above-cited references and publications are hereby incorporated by reference.

Equivalents

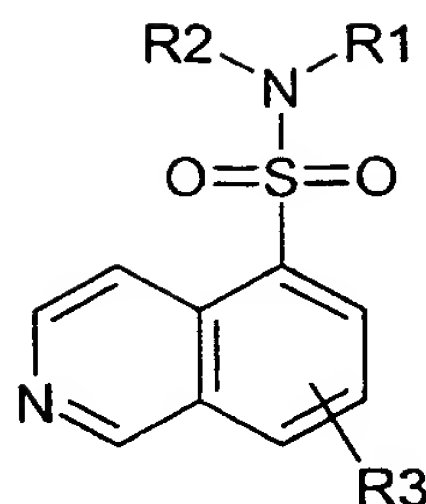
Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific polypeptides, nucleic acids, methods, assays and reagents described herein. Such equivalents are considered to be within the scope of this invention.

We claim:

- 5 1. A method for preventing degradation in functional performance of motor or sensory nerves in an animal comprising administering to the animal a therapeutic amount of a *hedgehog* or *ptc* therapeutic.
2. A method for preventing dysfunction of motor or sensory nerve cells comprising contacting the cells with an effective amount of a *hedgehog* or *ptc* therapeutic.
- 10 3. A method for treating or preventing peripheral neuroathy comprising administering to an animal a protective amount of a *hedgehog* or *ptc* therapeutic.
4. A method for protecting peripheral nerve cells under conditions which otherwise result in peripheral neuropathy, comprising administering to a patient in need thereof a therapeutically effective amount of a *hedgehog* or *ptc* therapeutic.
- 15 5. A method for the treating or preventing diabetic neuropathy comprising administering to a patient in need thereof a therapeutically effective amount of a *hedgehog* or *ptc* therapeutic.
6. A method for the treating or preventing virally-induced peripheral neuropathy comprising administering to a patient in need thereof a therapeutically effective amount of a *hedgehog* or *ptc* therapeutic.
- 20 7. The method of any of claims 1-6, wherein the *hedgehog* therapeutic is a polypeptide which includes a hedgehog amino acid sequence which is identical or homologous to an amino acid sequence of any one of SEQ ID Nos. 10-18.
8. The method of claim 7, wherein the hedgehog amino acid sequence is sufficient for specific binding of the polypeptide to a *patched* protein.
- 25 9. The method of claim 7, wherein the hedgehog amino acid sequence is at least 80 percent identical to an amino acid sequence of any one of SEQ ID Nos. 10-18.
10. The method of claim 7, wherein the hedgehog amino acid sequence is encodable by a nucleic acid which hybridizes under stringent conditions to any one of SEQ ID Nos. 1-9.
- 30 11. The method of claim 7, wherein the hedgehog amino acid sequence is of a vertebrate hedgehog protein.
12. The method of claim 11, wherein the vertebrate hedgehog protein is *Dhh*.

13. The method of claim 7, wherein the polypeptide includes at least a 50 amino acid extracellular portion of a vertebrate hedgehog protein.
14. The method of claim 7, wherein the polypeptide includes at least a 150 amino acid extracellular portion of a vertebrate hedgehog protein.
- 5 15. The method of claim 7, wherein the polypeptide includes at least an extracellular portion of a vertebrate hedgehog protein corresponding to residues 24-194 of SEQ ID No:15.
16. The method of claim 7, wherein the hedgehog polypeptide is modified with one or more lipophilic moieties.
- 10 17. The method of claim 16, wherein the hedgehog polypeptide is modified with one or more sterol moieties.
18. The method of claim 17, wherein the sterol moiety is cholesterol.
19. The method of claim 16, wherein the hedgehog polypeptide is modified with one or more fatty acid moieties.
- 15 20. The method of claim 19, wherein each fatty acid moiety is independently selected from the group consisting of myristoyl, palmitoyl, stearoyl, and arachidoyl.
21. The method of claim 16, wherein the hedgehog polypeptide is modified with one or more aromatic hydrocarbons.
22. The method of claim 21, wherein each aromatic hydrocarbon is ondependently
20 selected from the group consisting of benzene, perylene, phenanthrene, anthracene, naphthalene, pyrene, chrysene, and naphthacene.
23. The method of claim 16, wherein the hedgehog polypeptide is modified one or more times with a C7 - C30 alkyl or cycloalkyl.
24. The method of of any of claims 1-6, wherein the *ptc* therapeutic is a small organic
25 molecule.
25. The method of claim 24, wherein the binding of the *ptc* therapeutic to *patched* results in upregulation of patched and/or gli expression.
26. The method of any of claims 1-6, wherein the *ptc* therapeutic binds to *patched* and mimics *hedgehog*-mediated *patched* signal transduction.
- 30 27. The method of claim 26, wherein the *ptc* therapeutic is a small organic molecule.
28. The method of claim 26, wherein the binding of the *ptc* therapeutic to *patched* results in upregulation of patched and/or gli expression.

29. The method of any of claims 1-6, wherein the *ptc* therapeutic is a small organic molecule which interacts with neuronal cells to mimic *hedgehog*-mediated *patched* signal transduction.
- 5 30. The method of any of claims 1-6, wherein the *ptc* therapeutic mimics *hedgehog*-mediated *patched* signal transduction by altering the localization, protein-protein binding and/or enzymatic activity of an intracellular protein involved in a *patched* signal pathway.
- 10 31. The method of any of claims 1-6, wherein the *ptc* therapeutic alters the level of expression of a *hedgehog* protein, a *patched* protein or a protein involved in the intracellular signal transduction pathway of *patched*.
32. The method of claim 31, wherein the *ptc* therapeutic is an antisense construct which inhibits the expression of a protein which is involved in the signal transduction pathway of *patched* and the expression of which antagonizes *hedgehog*-mediated signals.
- 15 33. The method of claim 32, wherein the antisense construct is an oligonucleotide of about 20-30 nucleotides in length and having a GC content of at least 50 percent.
34. The method of claim 33, wherein the antisense oligonucleotide is selected from the group consisting of: 5'-GTCCTGGCGCCGCCGCCGCCGTCGCC;
5'-TTCCGATGACCGGCCTTTCGCGGTGA; and
20 5'-GTGCACGGAAAGGTGCAGGCCACACT
35. The method of claims 31, wherein the *ptc* therapeutic is a small organic molecule which binds to *patched* and regulates *patched*-dependent gene expression.
36. The method of claim 35, wherein the *ptc* therapeutic is an inhibitor of protein kinase A.
- 25 37. The method of claim 36, wherein the PKA inhibitor is a 5-isoquinolinesulfonamide
38. The method of claim 37, wherein the PKA inhibitor is represented in the general formula:



wherein,

R_1 and R_2 each can independently represent hydrogen, and as valence and stability permit a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-R_8$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$ -lower alkyl, $-(CH_2)_m-O$ -lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$ -lower alkyl, $-(CH_2)_m-S$ -lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$, or

R_1 and R_2 taken together with N form a heterocycle (substituted or unsubstituted);

R_3 is absent or represents one or more substitutions to the isoquinoline ring such as a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-R_8$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$ -lower alkyl, $-(CH_2)_m-O$ -lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$ -lower alkyl, $-(CH_2)_m-S$ -lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$;

R_8 represents a substituted or unsubstituted aryl, aralkyl, cycloalkyl, cycloalkenyl, or heterocycle; and

n and m are independently for each occurrence zero or an integer in the range of 1 to 6.

39. The method of claim 36, wherein the PKA inhibitor is cyclic AMP analog.

40. The method of claim 36, wherein the PKA inhibitor is selected from the group consisting of N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine, KT5720, 8-bromo-cAMP, dibutyryl-cAMP and PKA Heat Stable Inhibitor isoform α .

41. The method of any of claims 4-6, wherein patient is being treated prophylactically.

42. A therapeutic preparation of a small molecule antagonist of *patched*, which *patched* antagonist is provided in a pharmaceutically acceptable carrier and in an amount sufficient to treat a peripheral neuropathy.
- 5 43. A method for protecting peripheral nerve cells under conditions which otherwise result in peripheral neuropathy, comprising administering to a patient a gene activation construct which recombines with a genomic *hedgehog* gene of the patient to provide a heterologous transcriptional regulatory sequence operatively linked to a coding sequence of the *hedgehog* gene.
- 10 44. The method of claim 4, 5, 6 or 43, which method is part of a protocol for the treatment of an acquired neuropathy.
45. The method of claim 44, wherein the neuropathy is due to viral infection, diabetes or inflammation.
46. The method of claim 44, wherein the neuropathy is due to contact with a toxic agent.
- 15 47. The method of claim 44, wherein the neuropathy is selected from the group consisting of diabetic neuropathy; immune-mediated neuropathy, chronic inflammatory demyelinating polyneuropathy (CIDP), chronic polyneuropathy with antibodies to peripheral nerves, neuropathies associated with vasculitis or inflammation of the blood vessels in peripheral nerve, brachial or lumbosacral plexitis, and neuropathies associated with monoclonal gammopathies; neuropathies associated with tumors or neoplasms such as sensory neuropathy associated with lung cancer, neuropathy associated with multiple myeloma, neuropathy associated with waldenstrom's macroglobulemia, chronic lymphocytic leukemia, or B-cell lymphoma; neuropathy associated with amyloidosis; 20 neuropathies caused by infections; neuropathies caused by nutritional imbalance; neuropathy in kidney disease; hypothyroid neuropathy; neuropathy caused by alcohol and toxins; neuropathies caused by drugs; neuropathy resulting from local irradiation; neuropathies caused by trauma or compression; and idiopathic neuropathies 25
- 30 48. The method of claim 4, 5, 6 or 43, which method is part of a protocol for the treatment of a hereditary neuropathy.
49. The method of claim 48, whererin the neuropathy is selected from the group consisting of Charcot-Marie Tooth Disease (CMT); Familial Amyloidotic Neuropathy and Hereditary Porphyria.

50. The method of claim 4, 5, 6 or 43, which method is part of a protocol for slowing neurodegenerative events associated with age-related neuropathology.
51. The method of claim 7, wherein the hedgehog polypeptide is a fusion protein.

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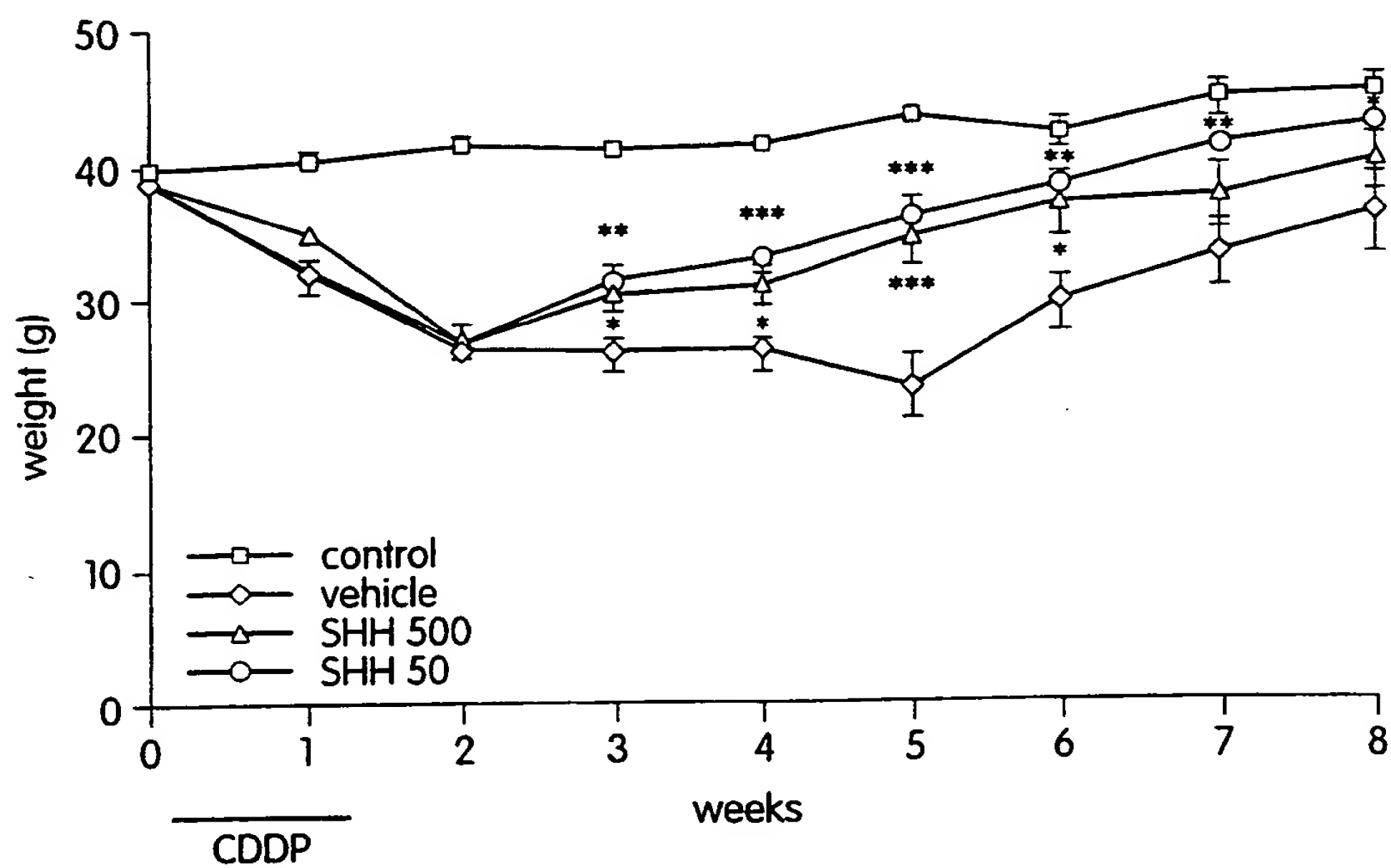


Fig. 1

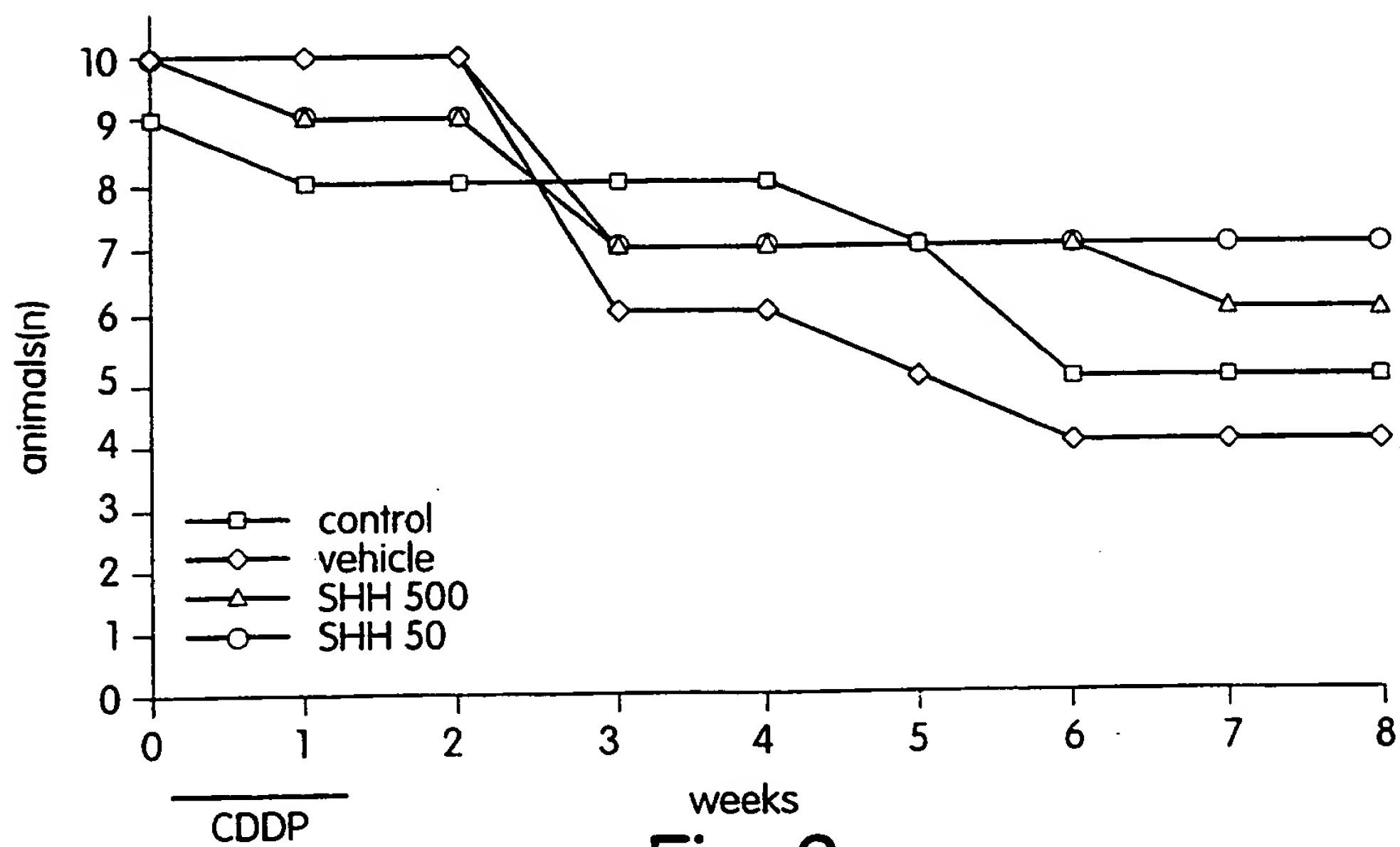


Fig. 2

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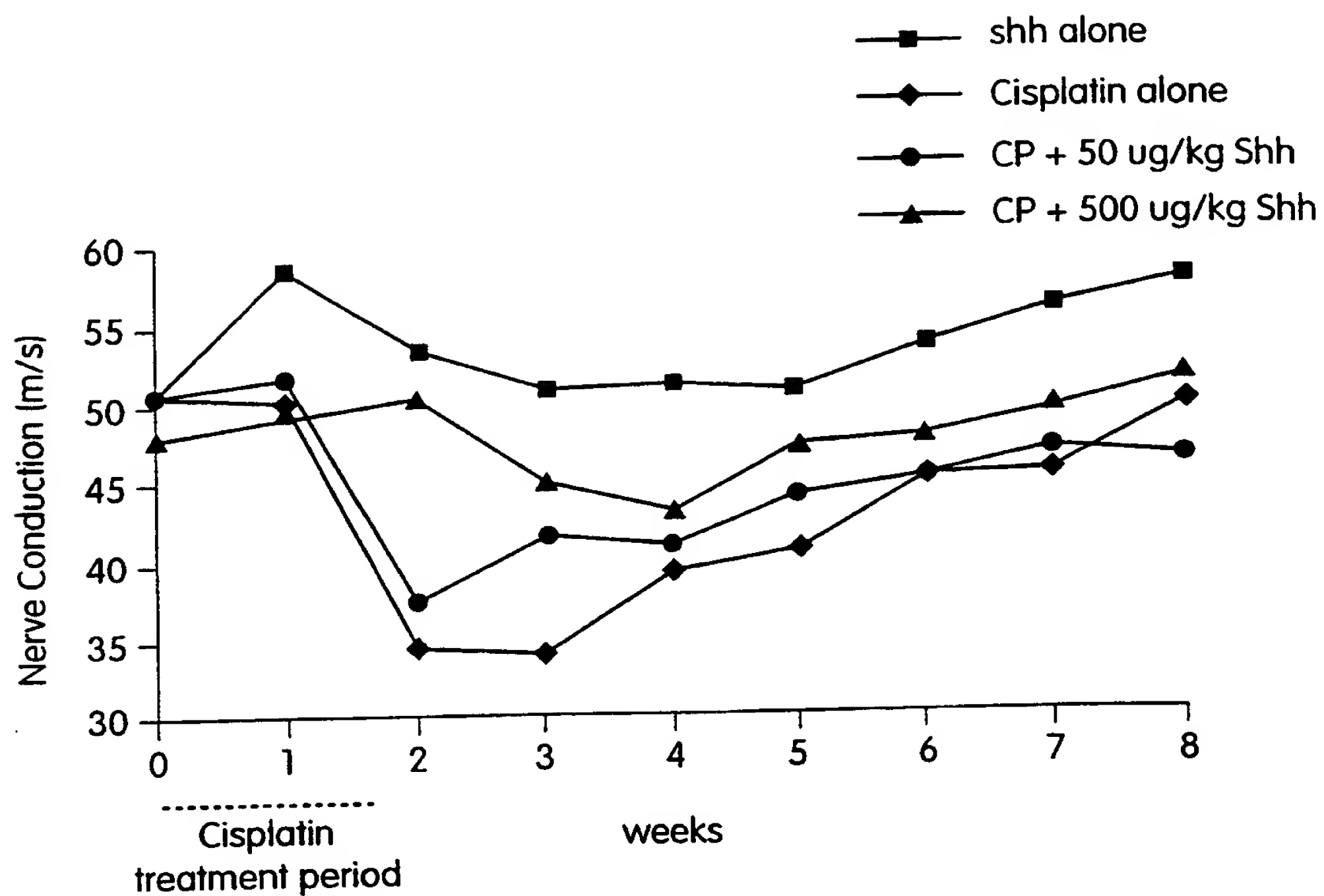


Fig. 3

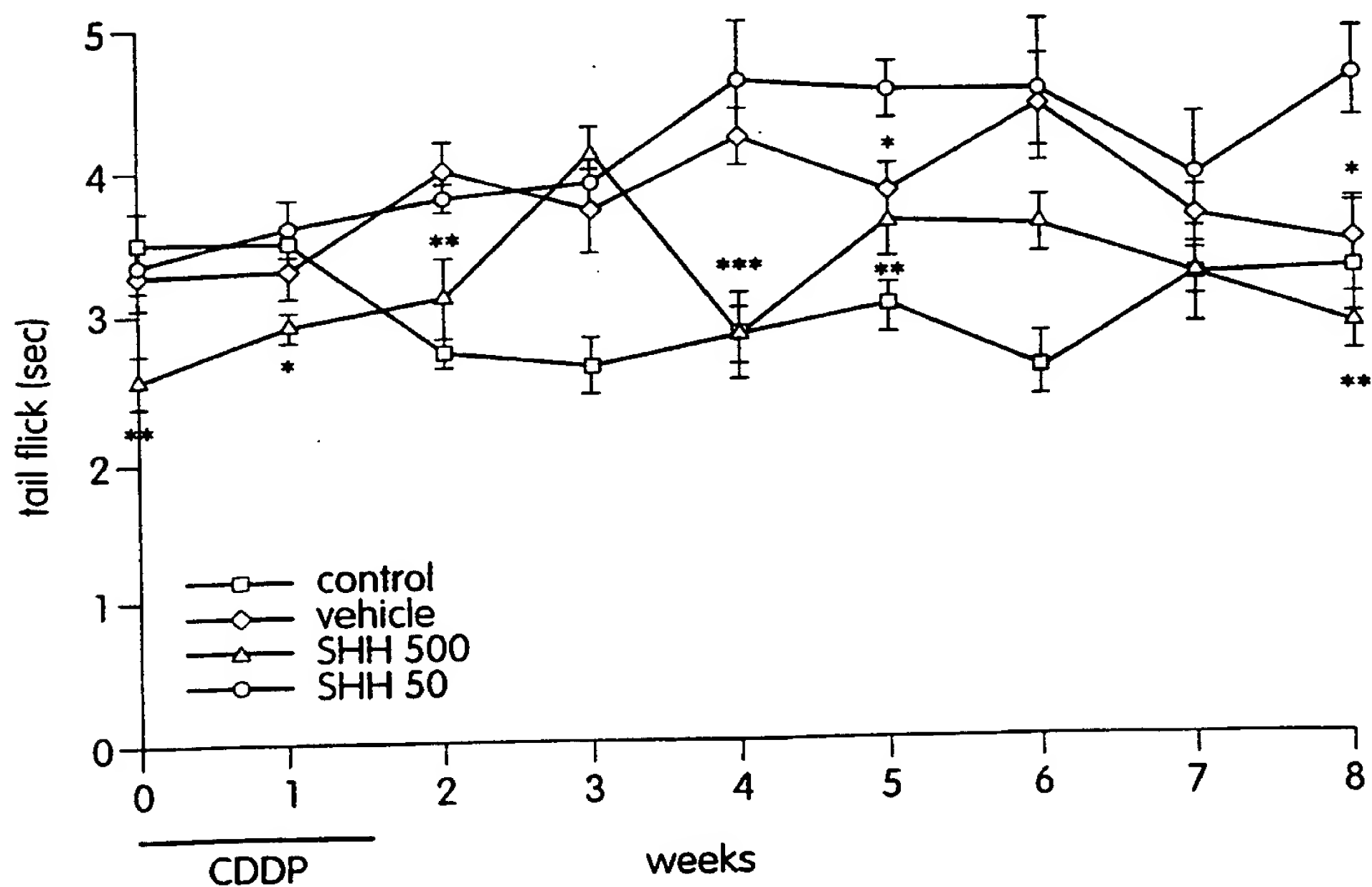


Fig. 4

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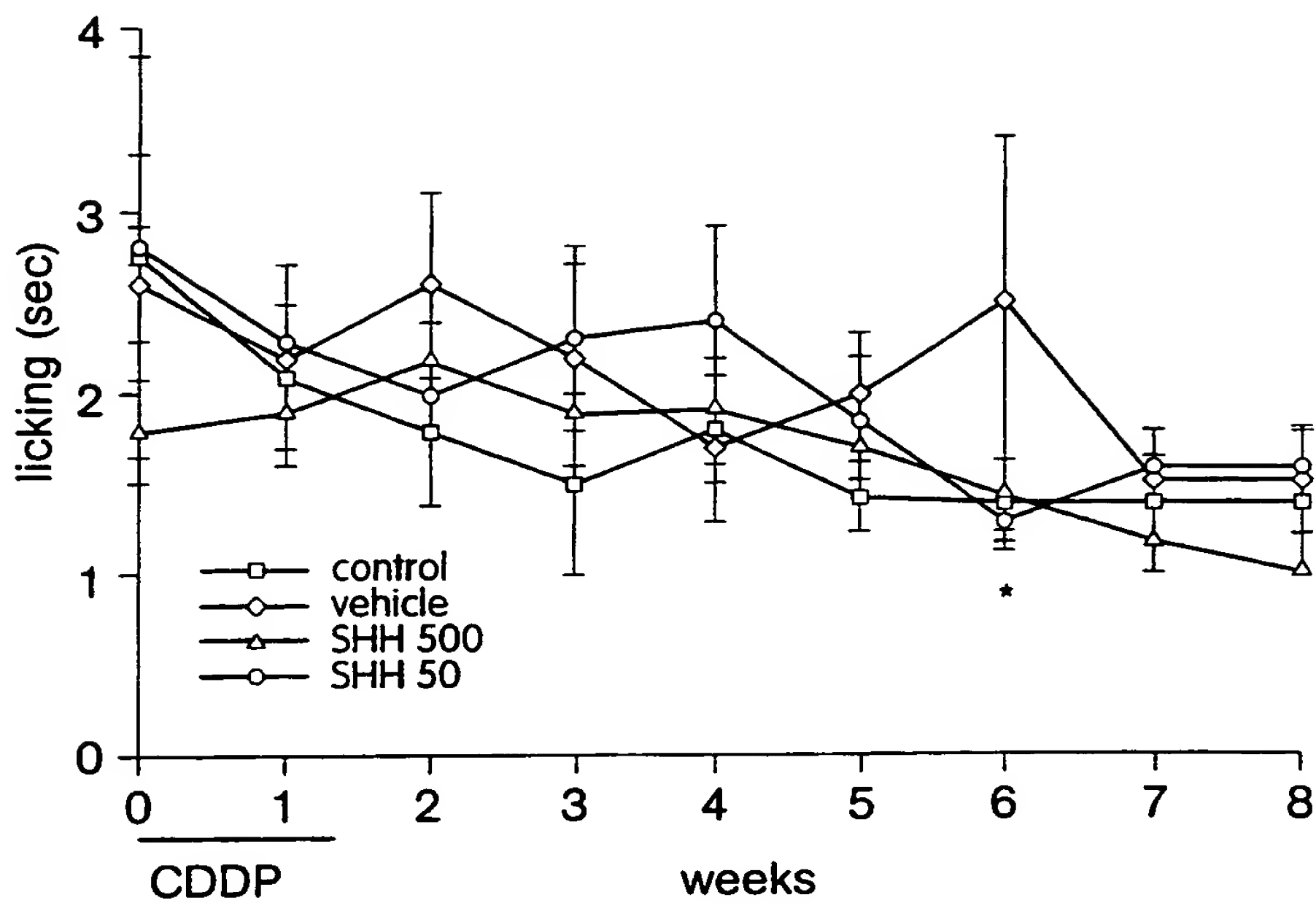


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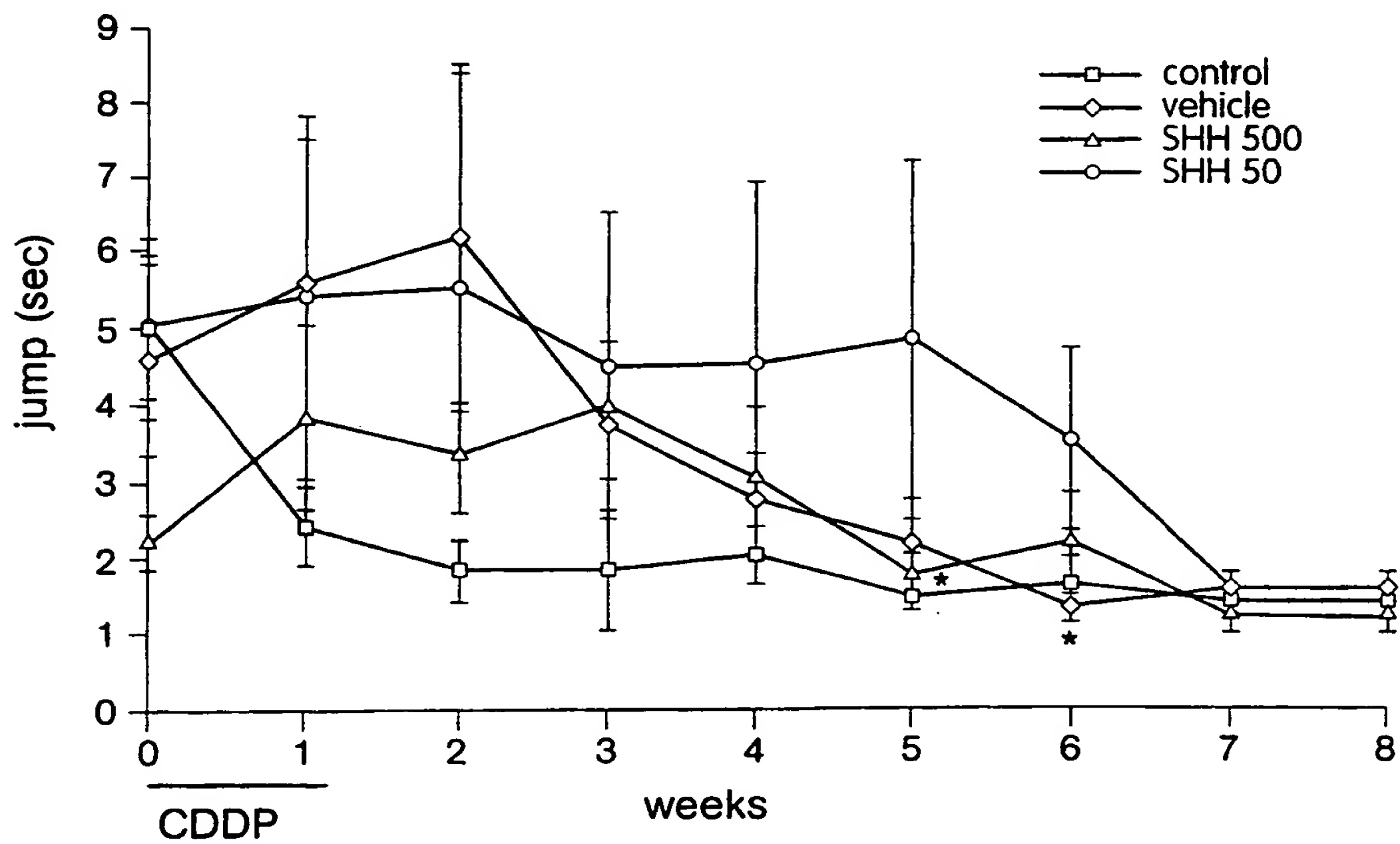


Fig. 6

SUBSTITUTE SHEET (RULE 26)

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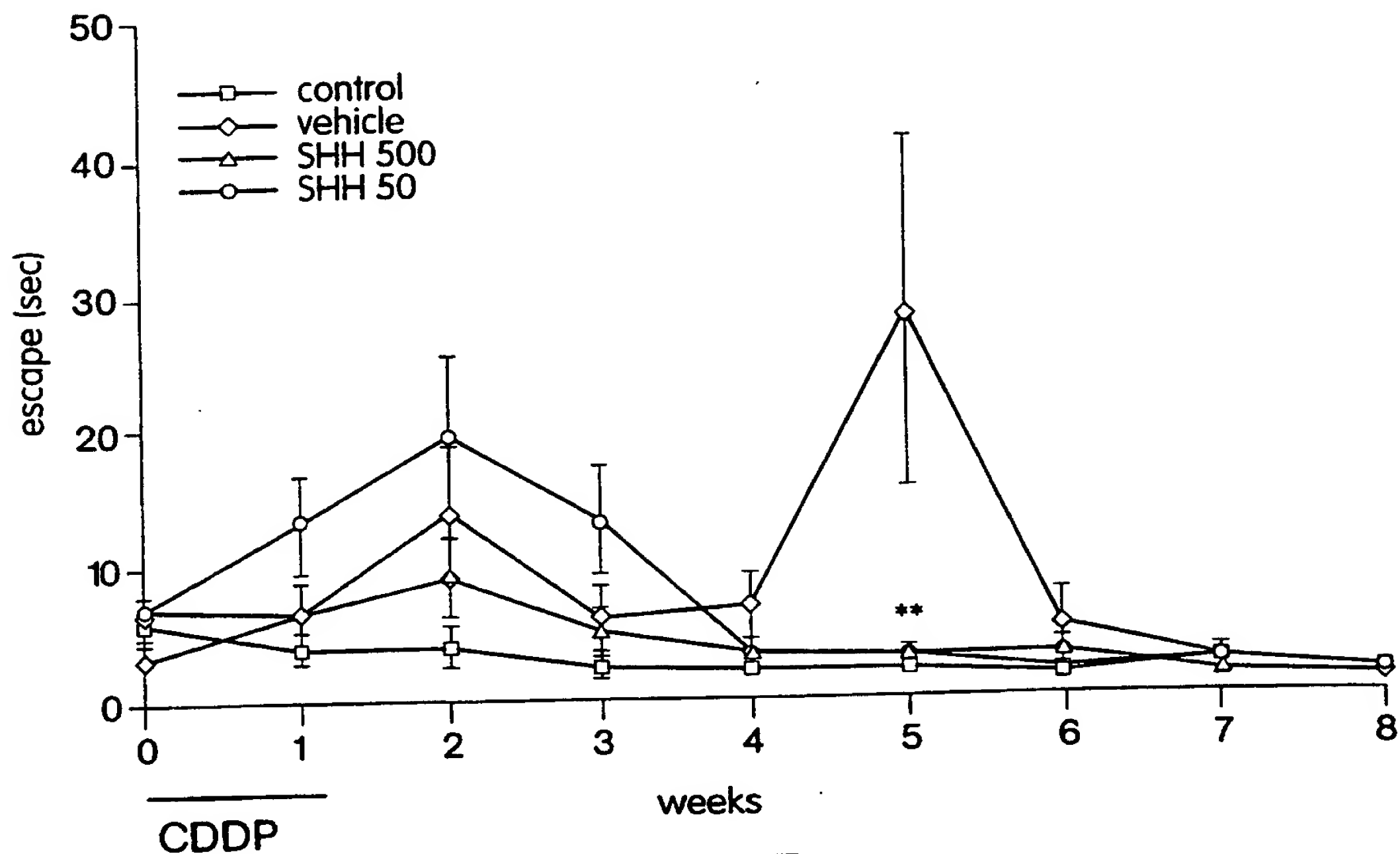


Fig. 7

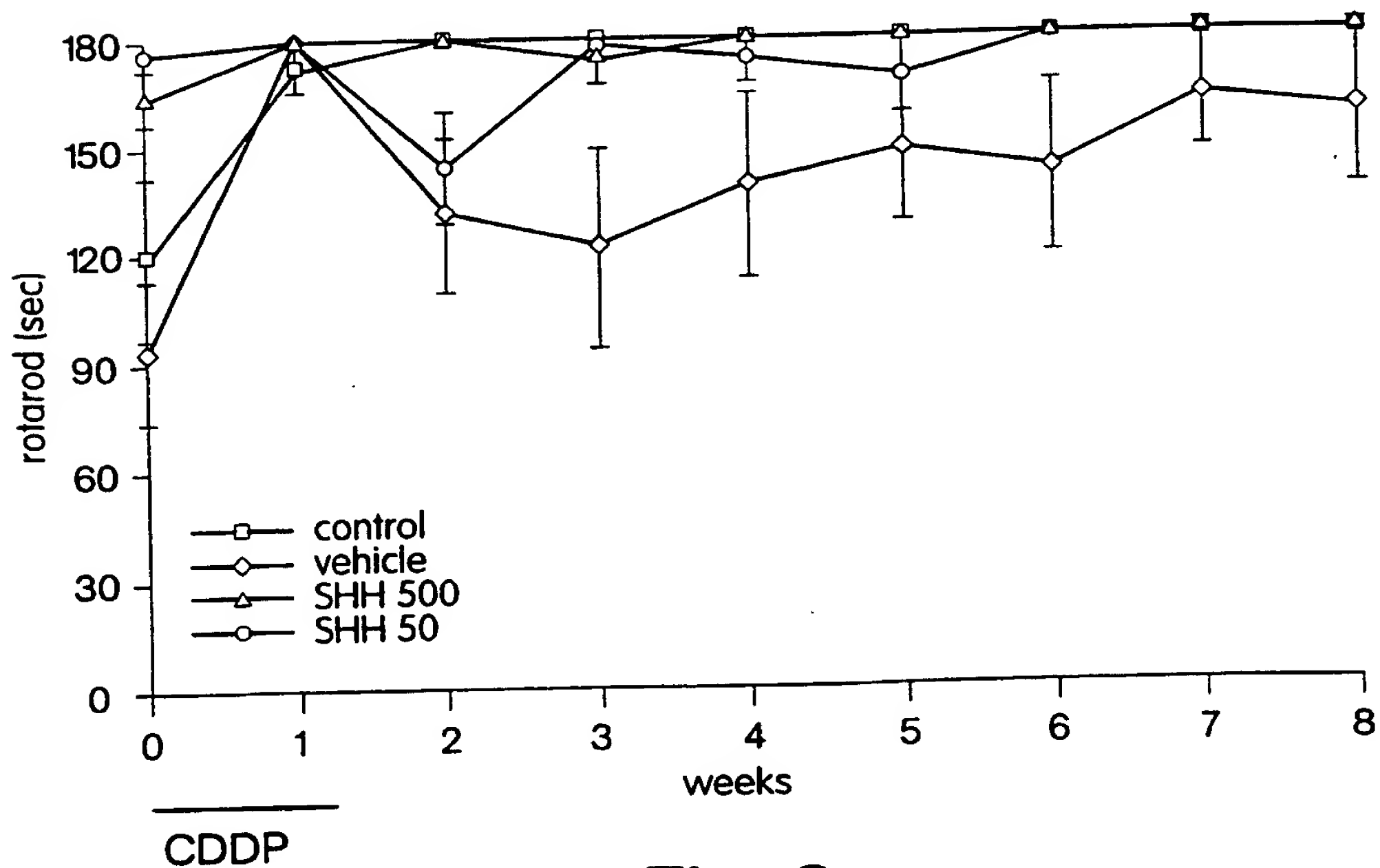


Fig. 8

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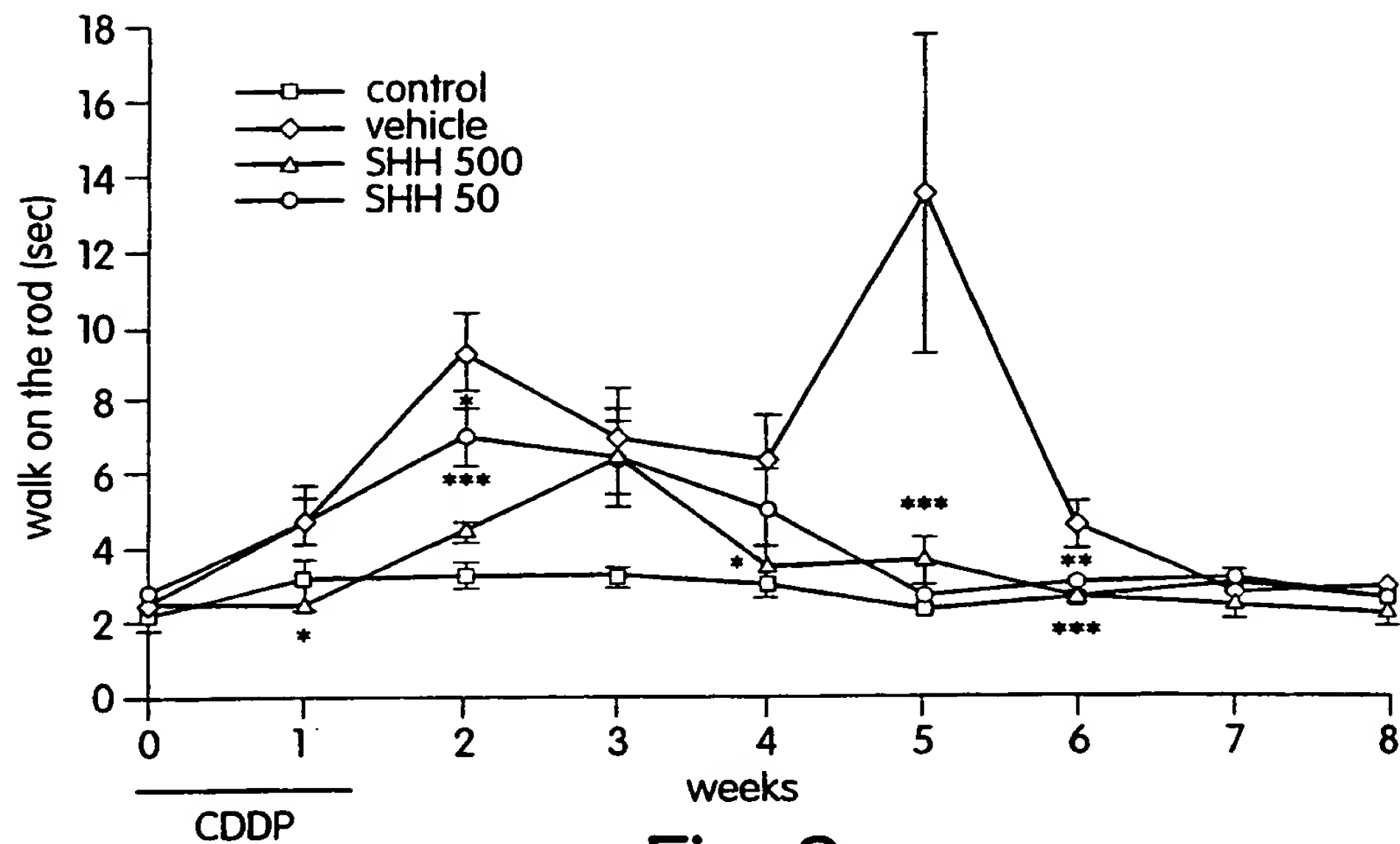


Fig. 9

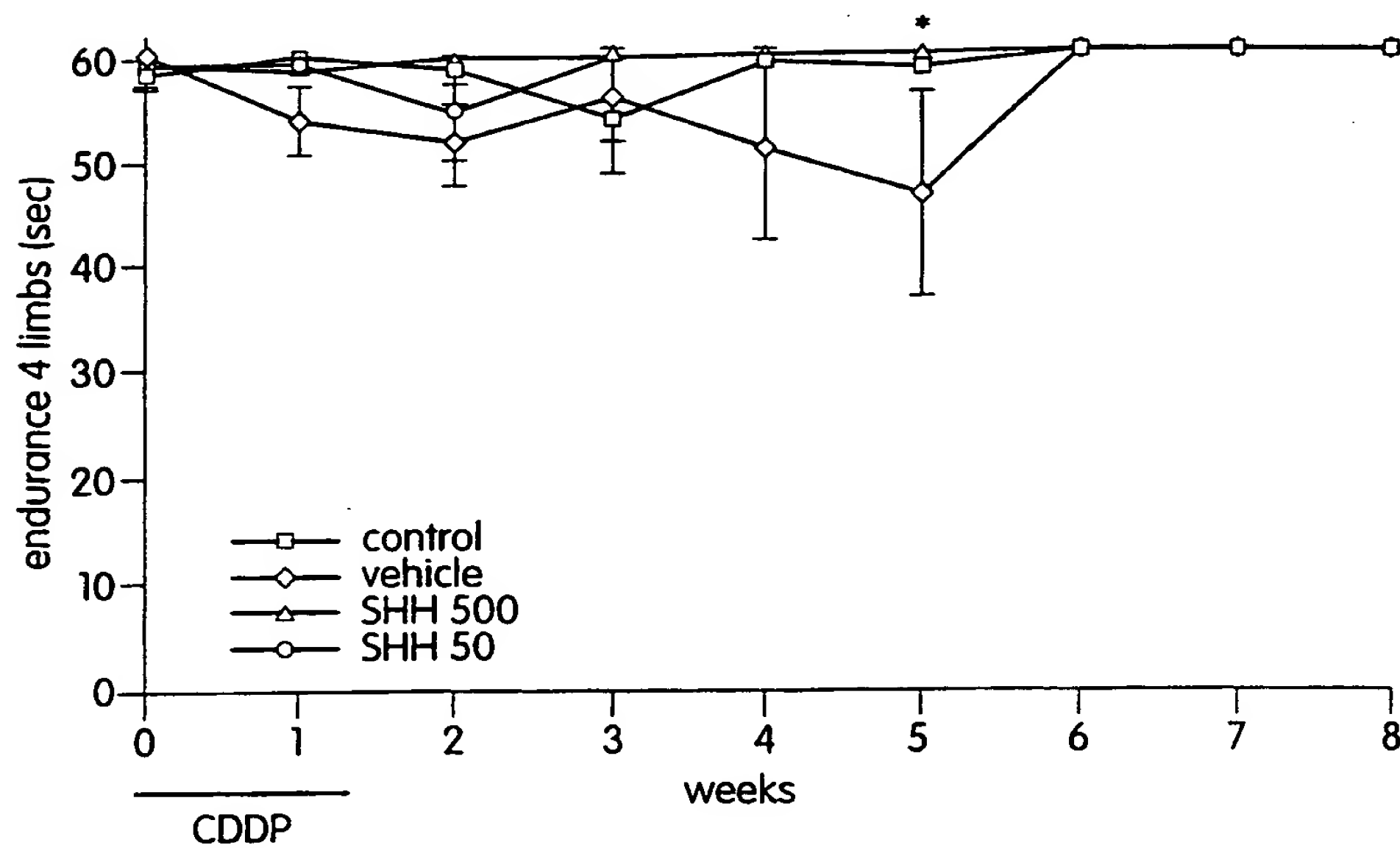


Fig. 10A

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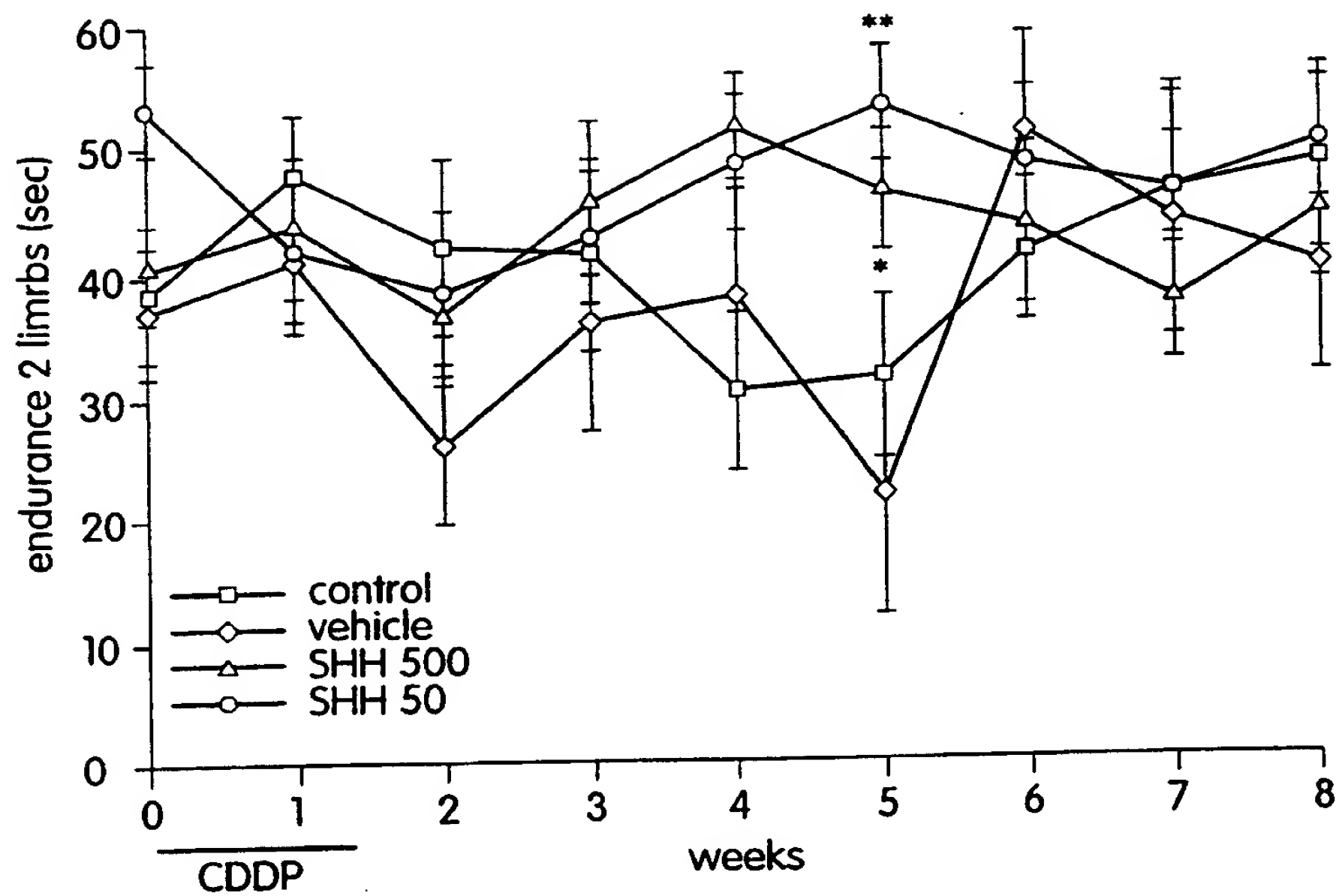


Fig. 10B

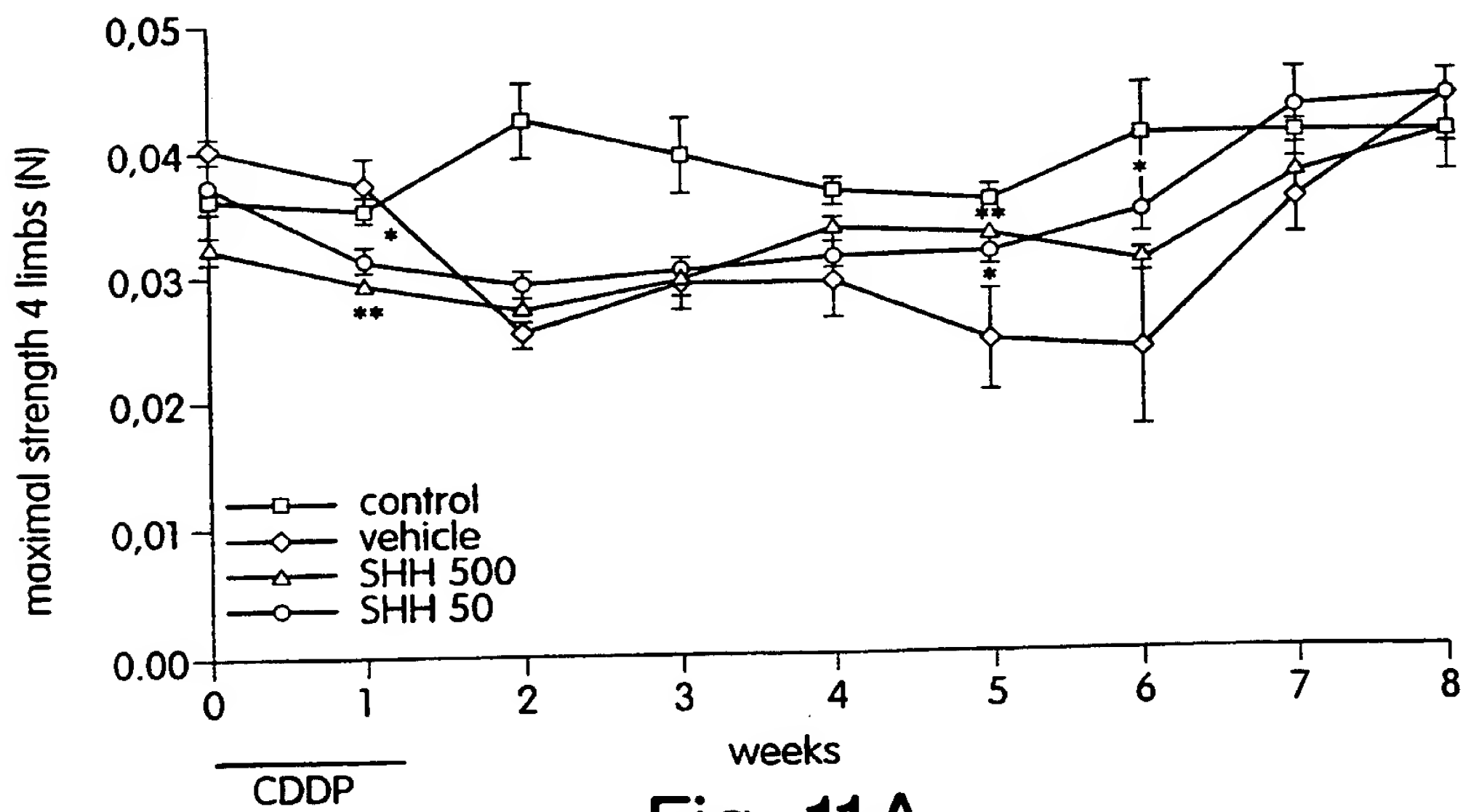


Fig. 11A

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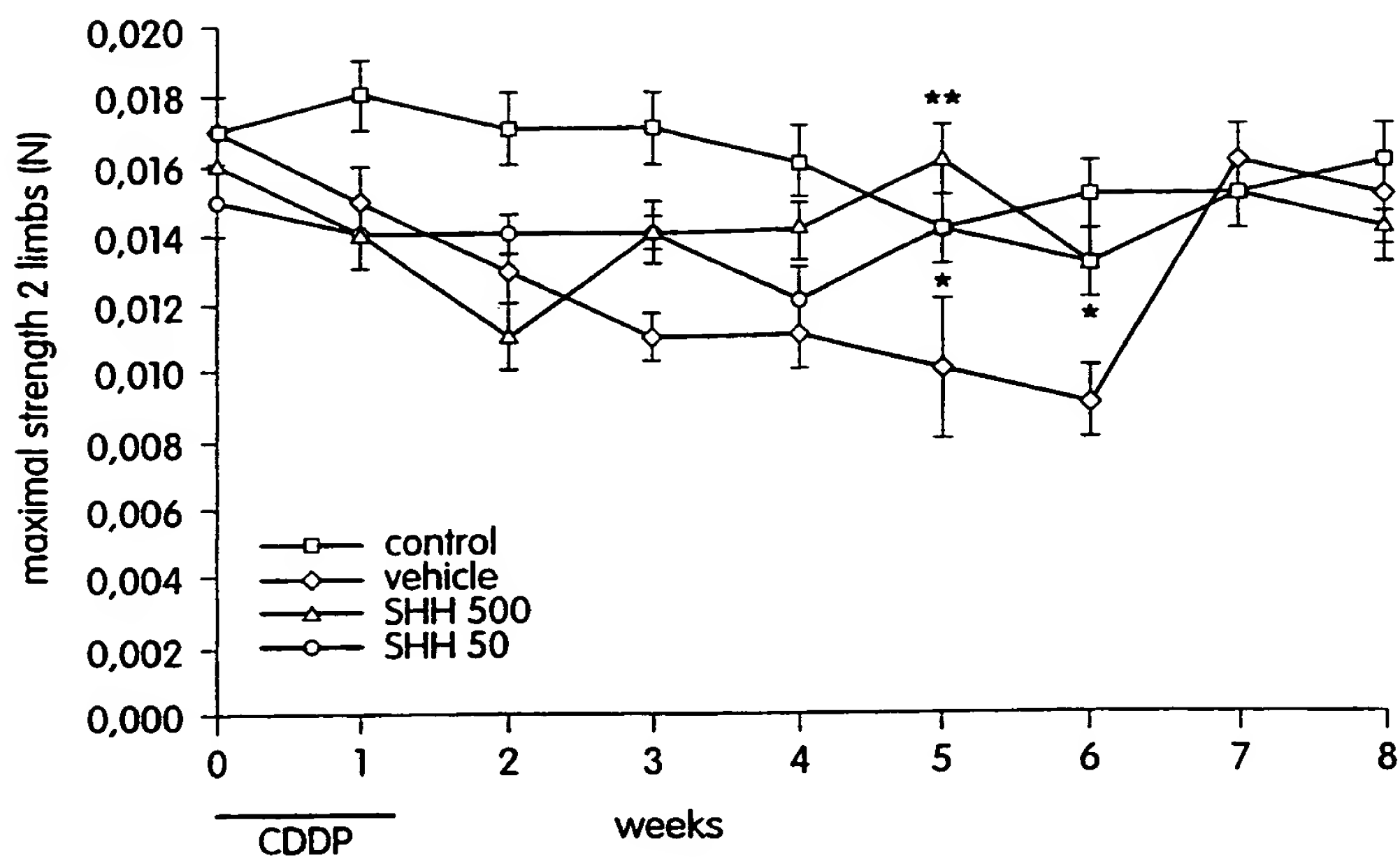


Fig. 11 B

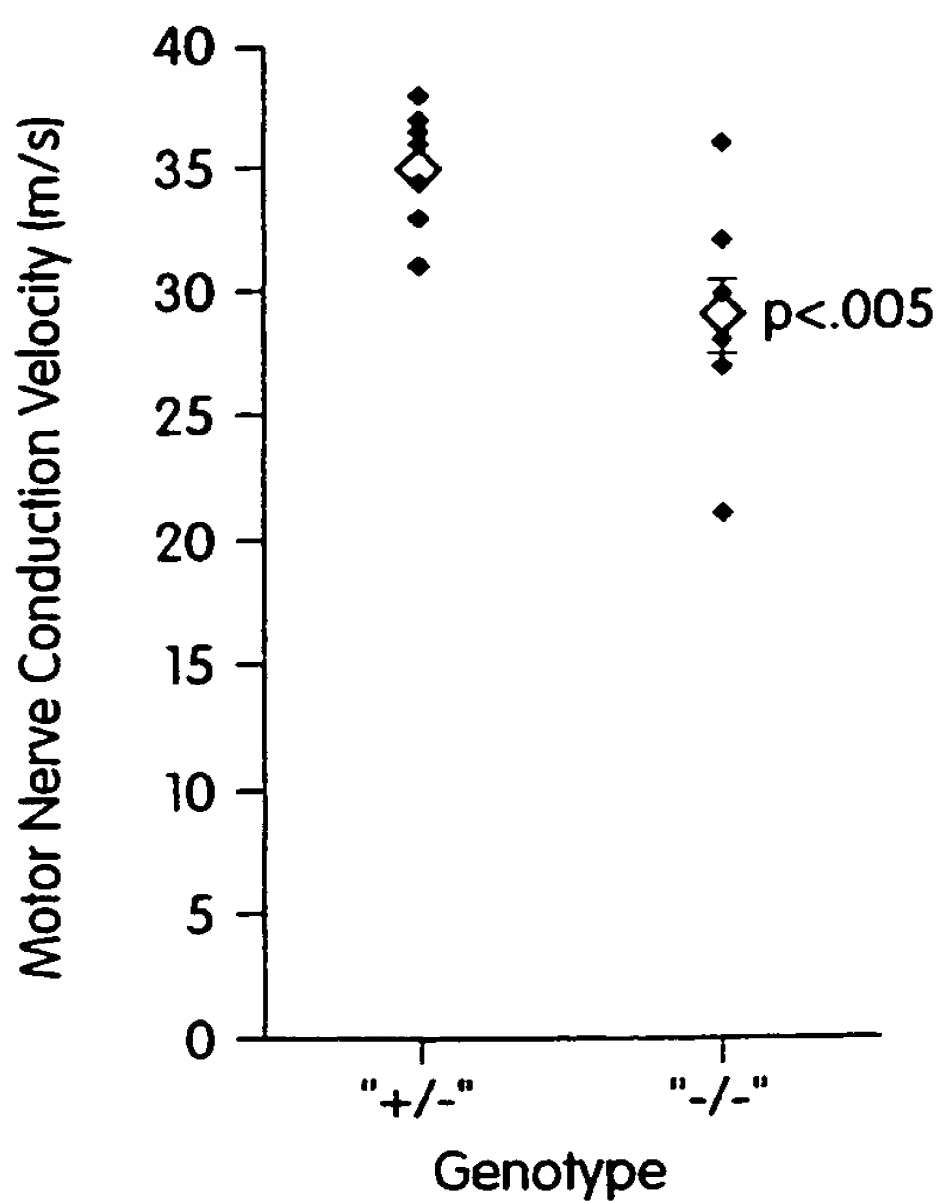


Fig. 12

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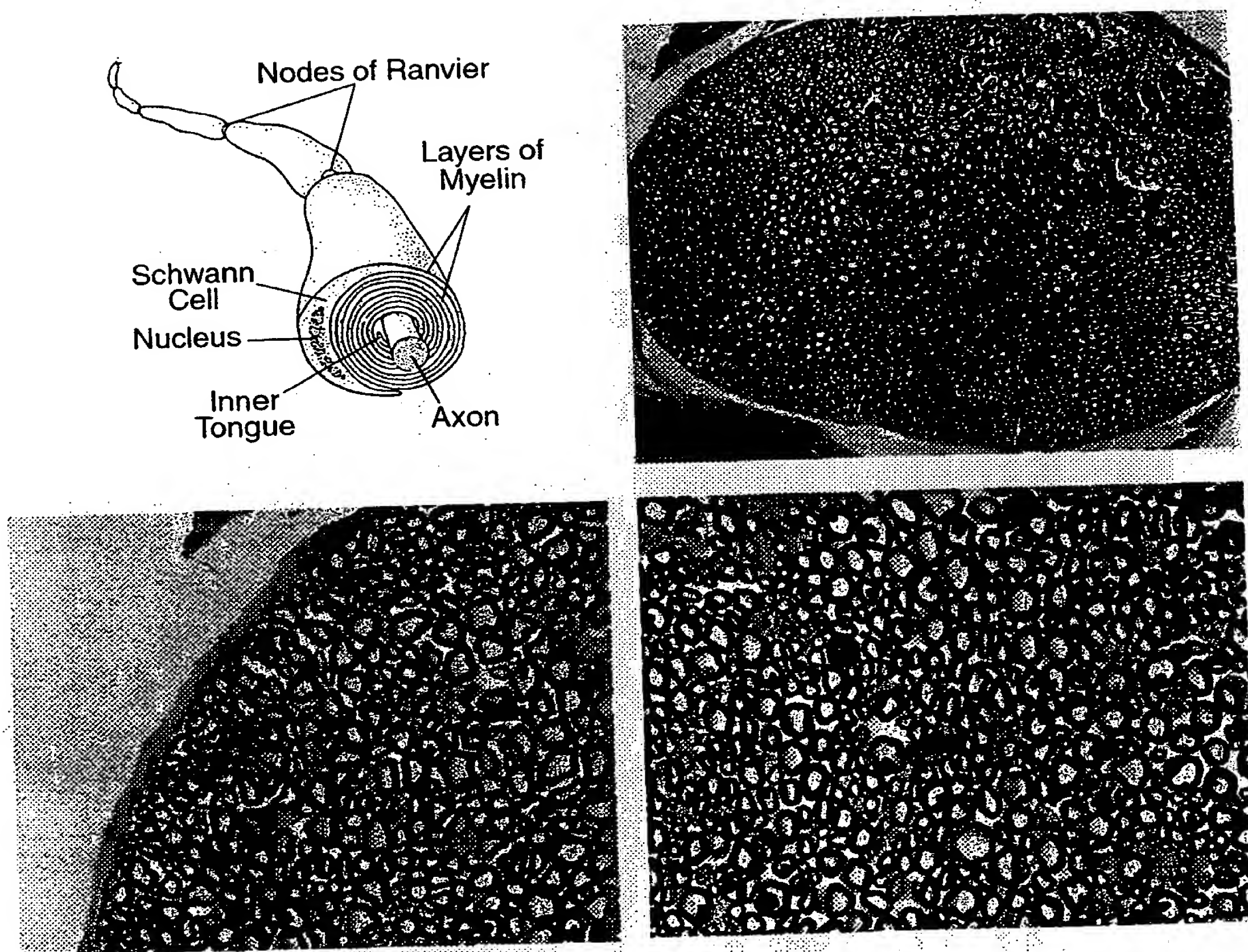


Fig. 13A

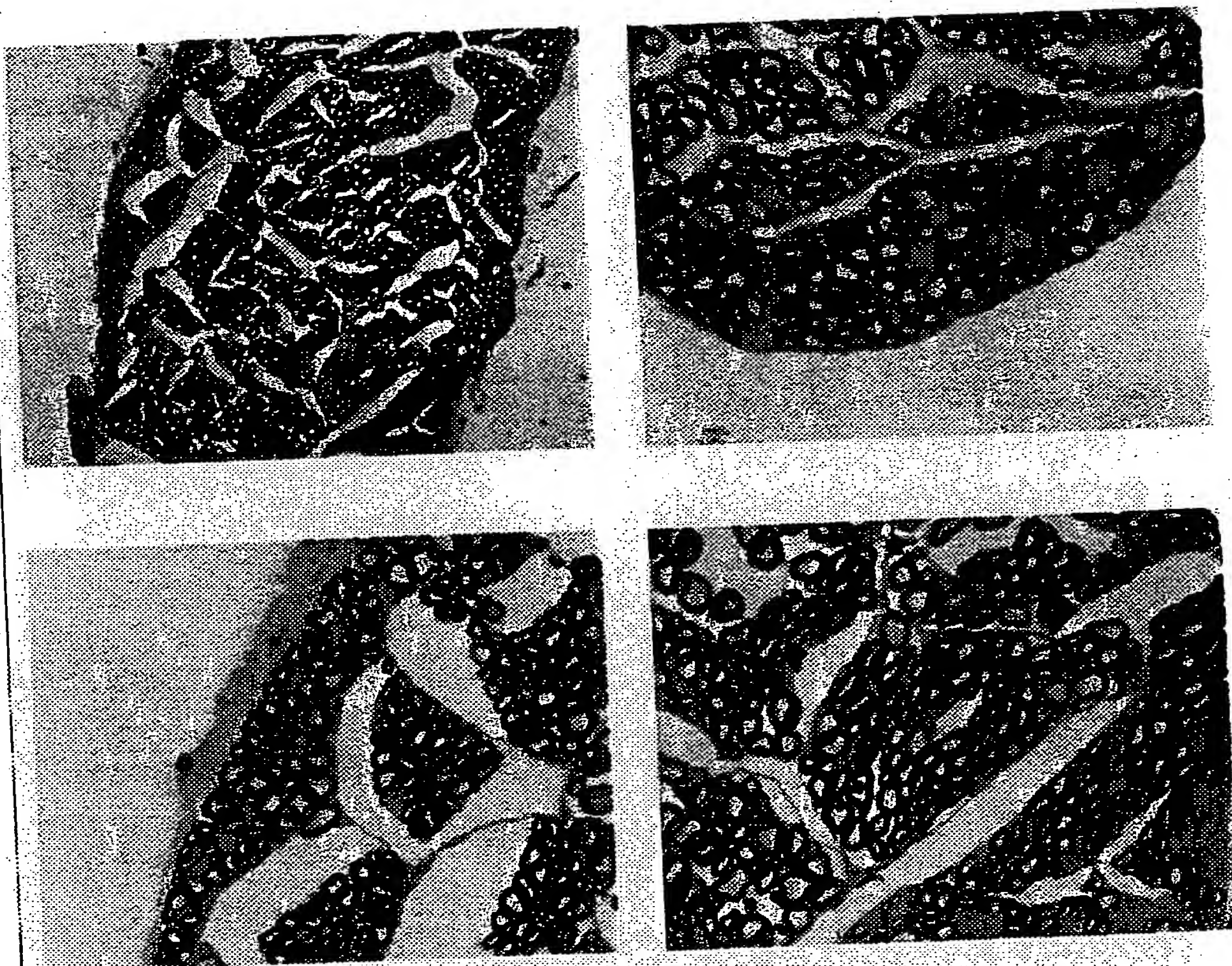


Fig. 13B

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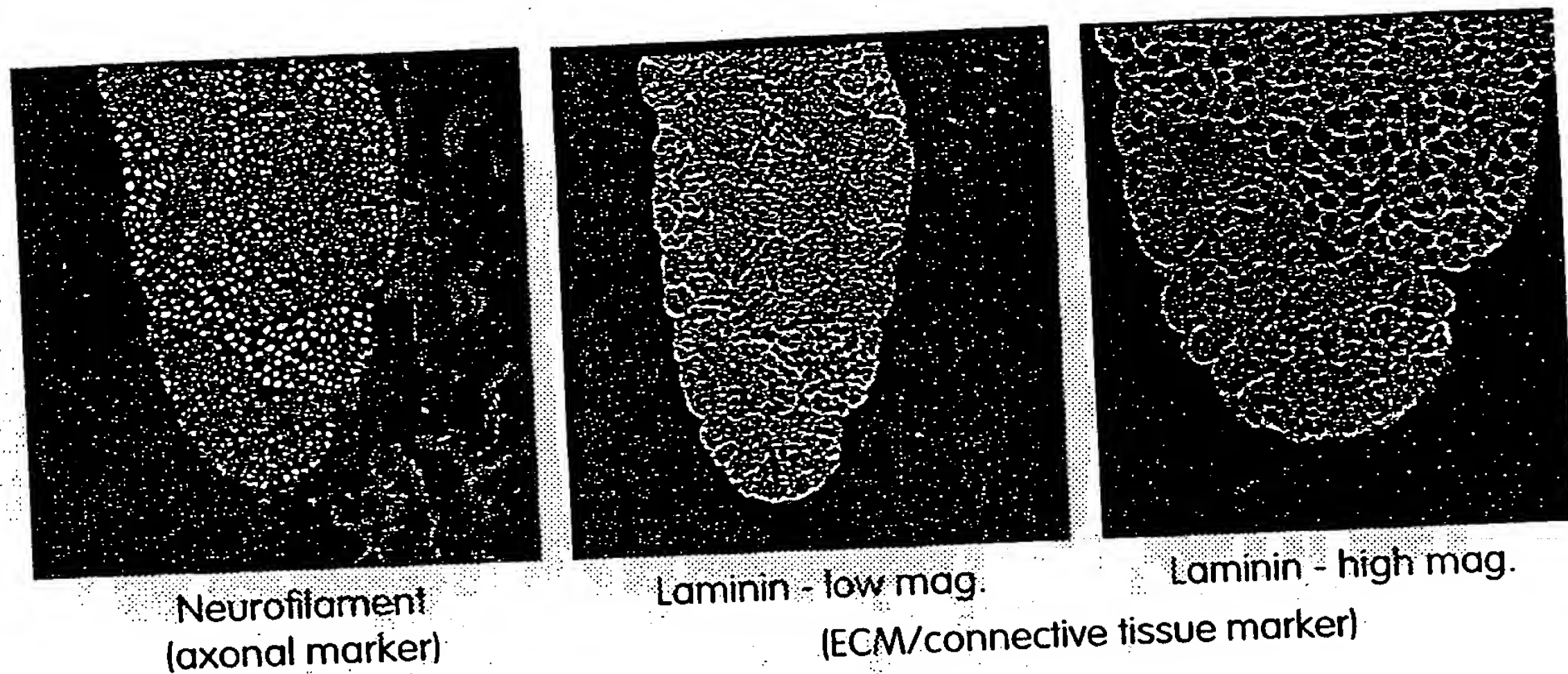


Fig. 14A

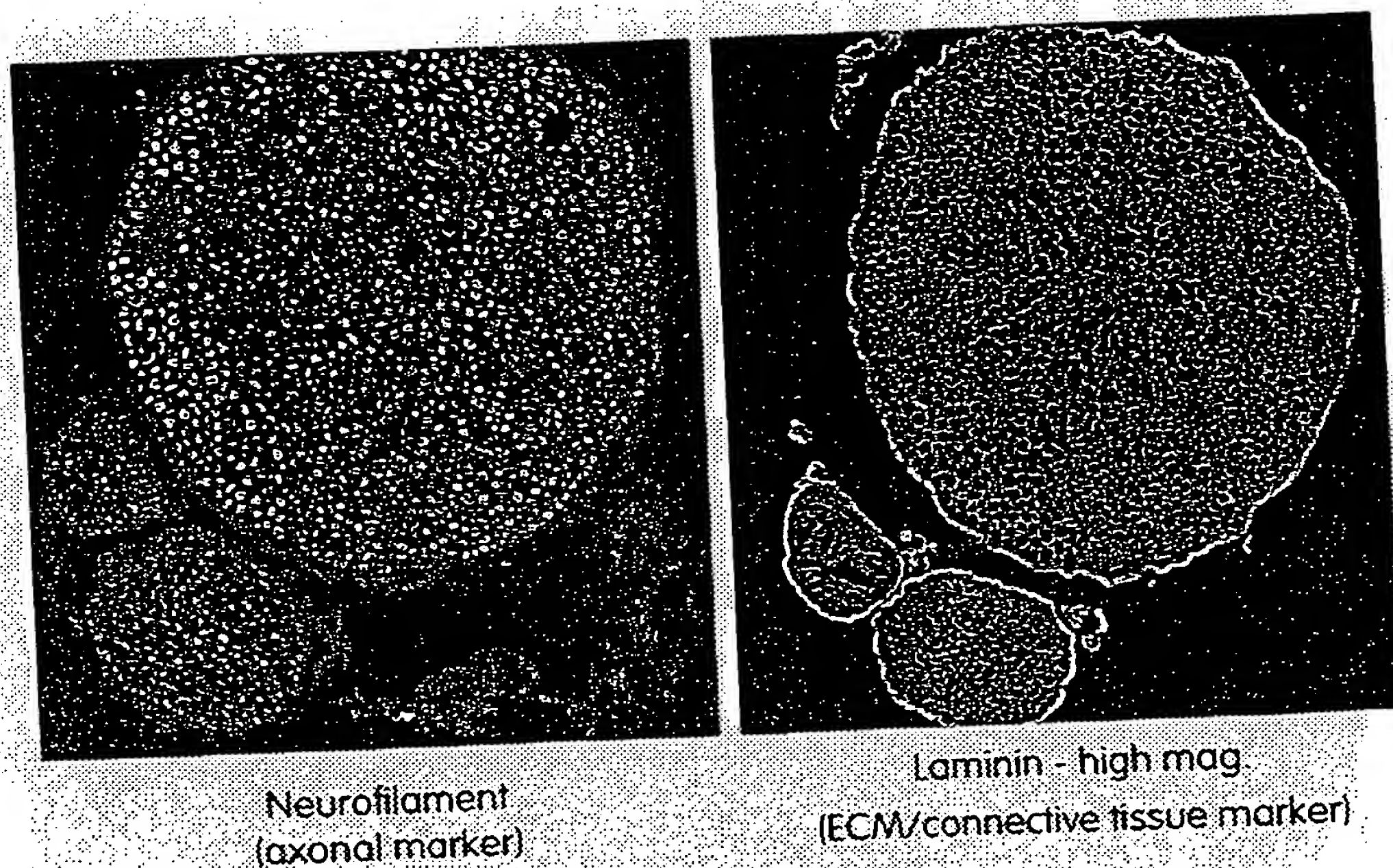
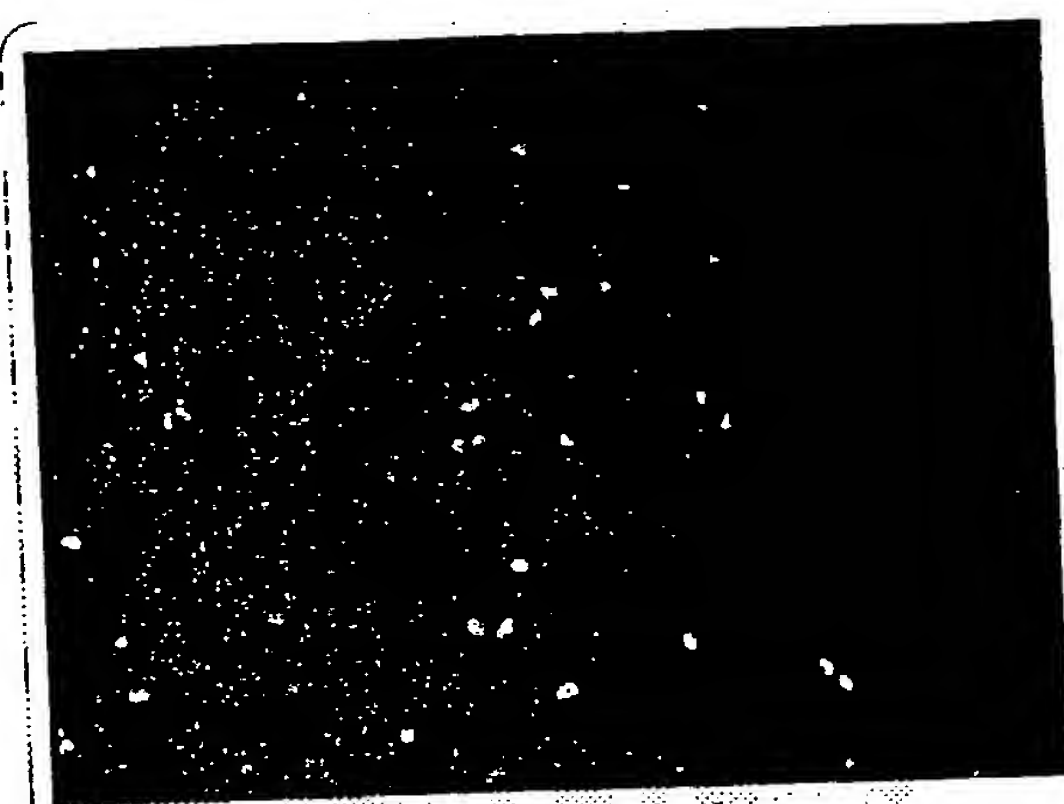


Fig. 14B

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- Dissociate P4 sciatic nerve
- Plate cells overnight
- Add Hh's and BrdU
- Fix 18-24 hours later
- Score cells

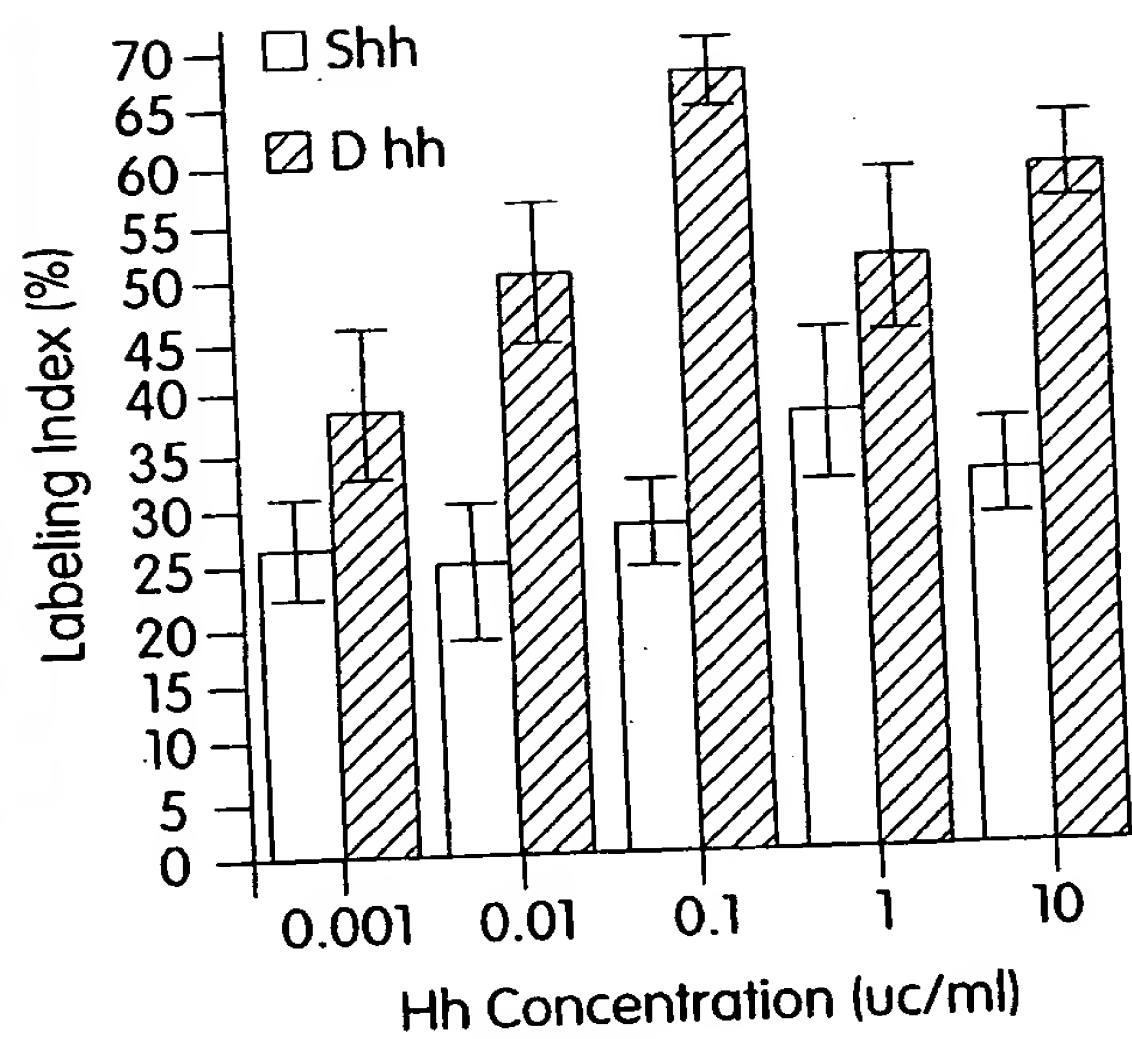


Fig. 15

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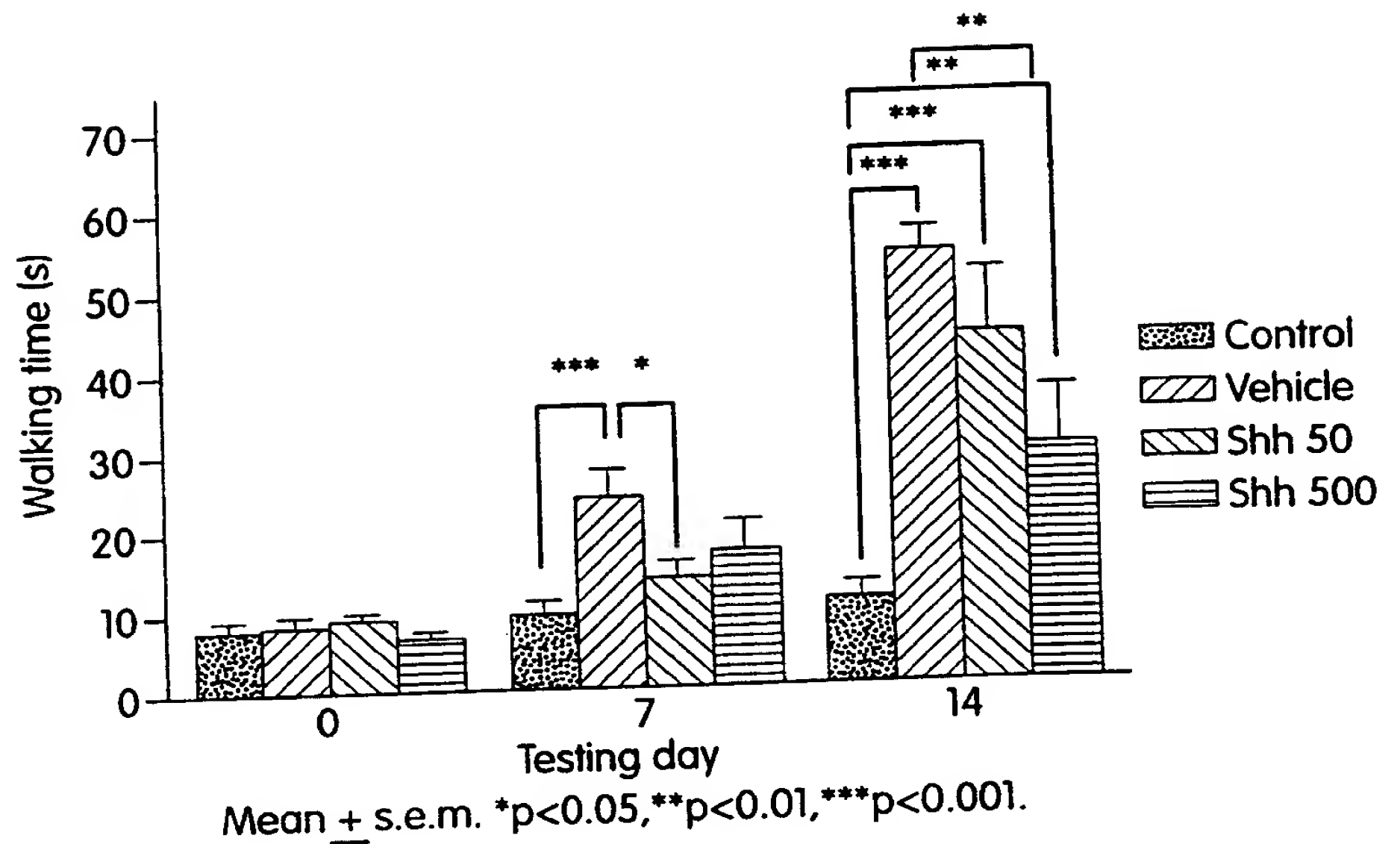


Fig. 16

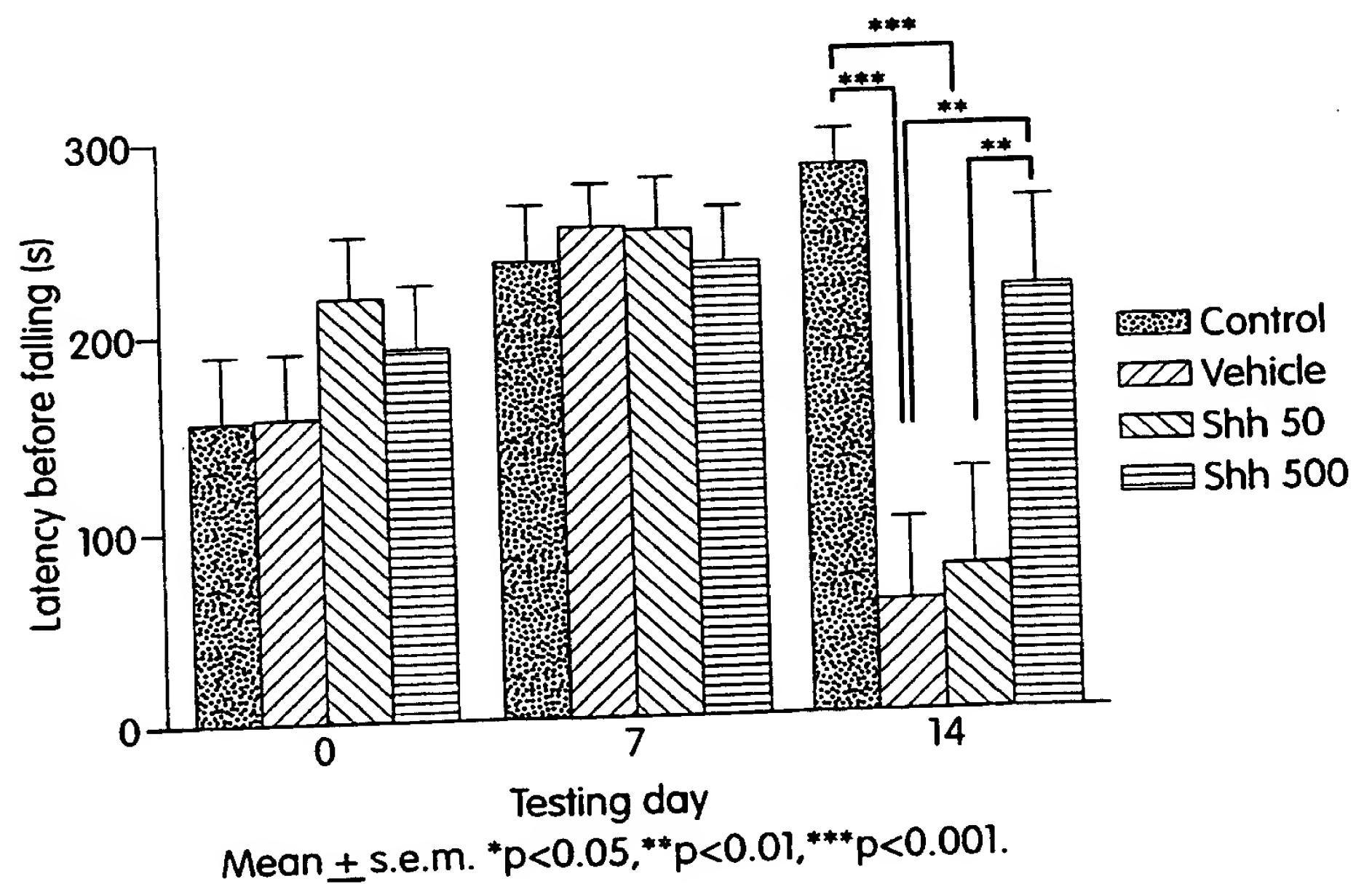


Fig. 17

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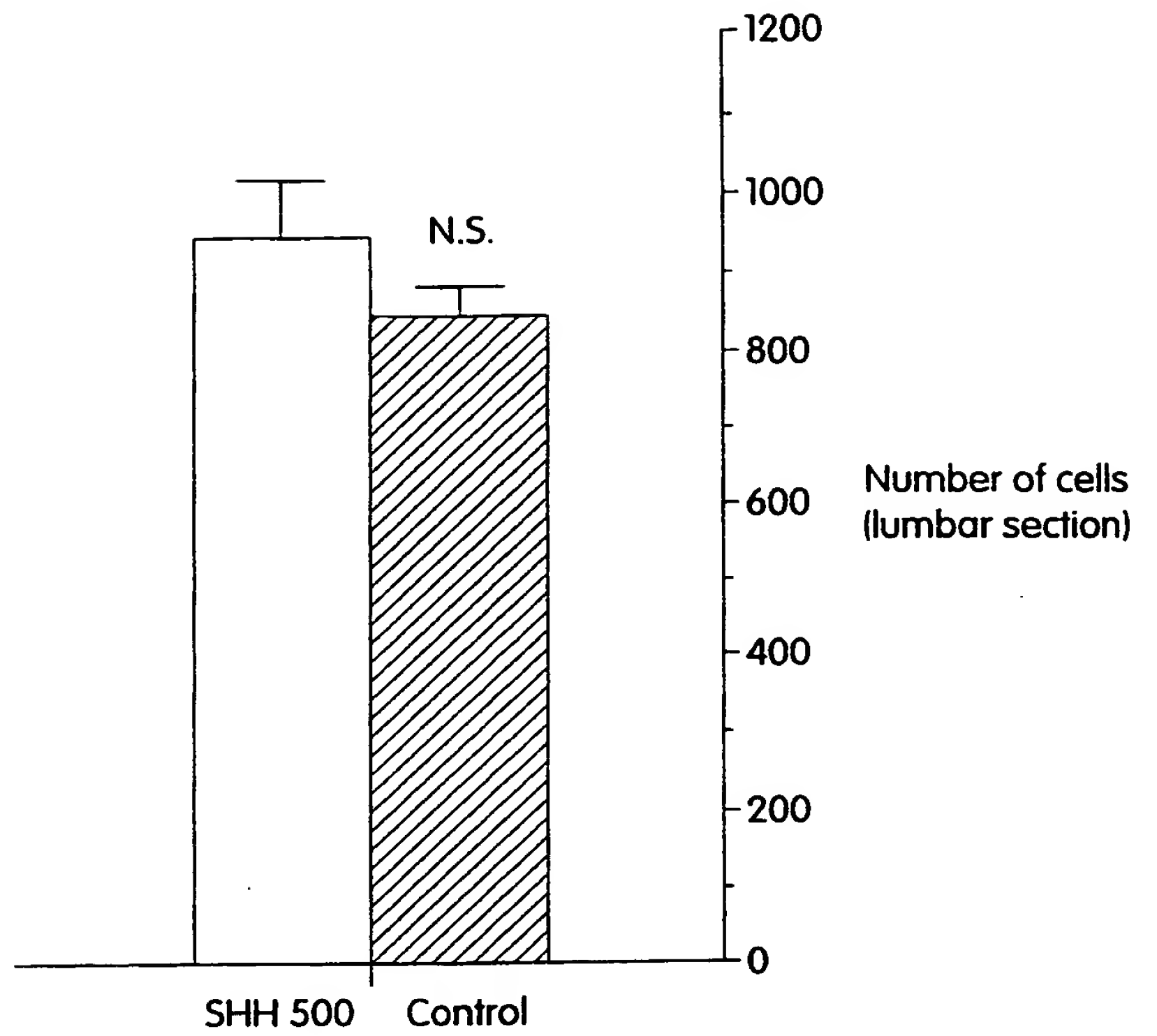


Fig. 18

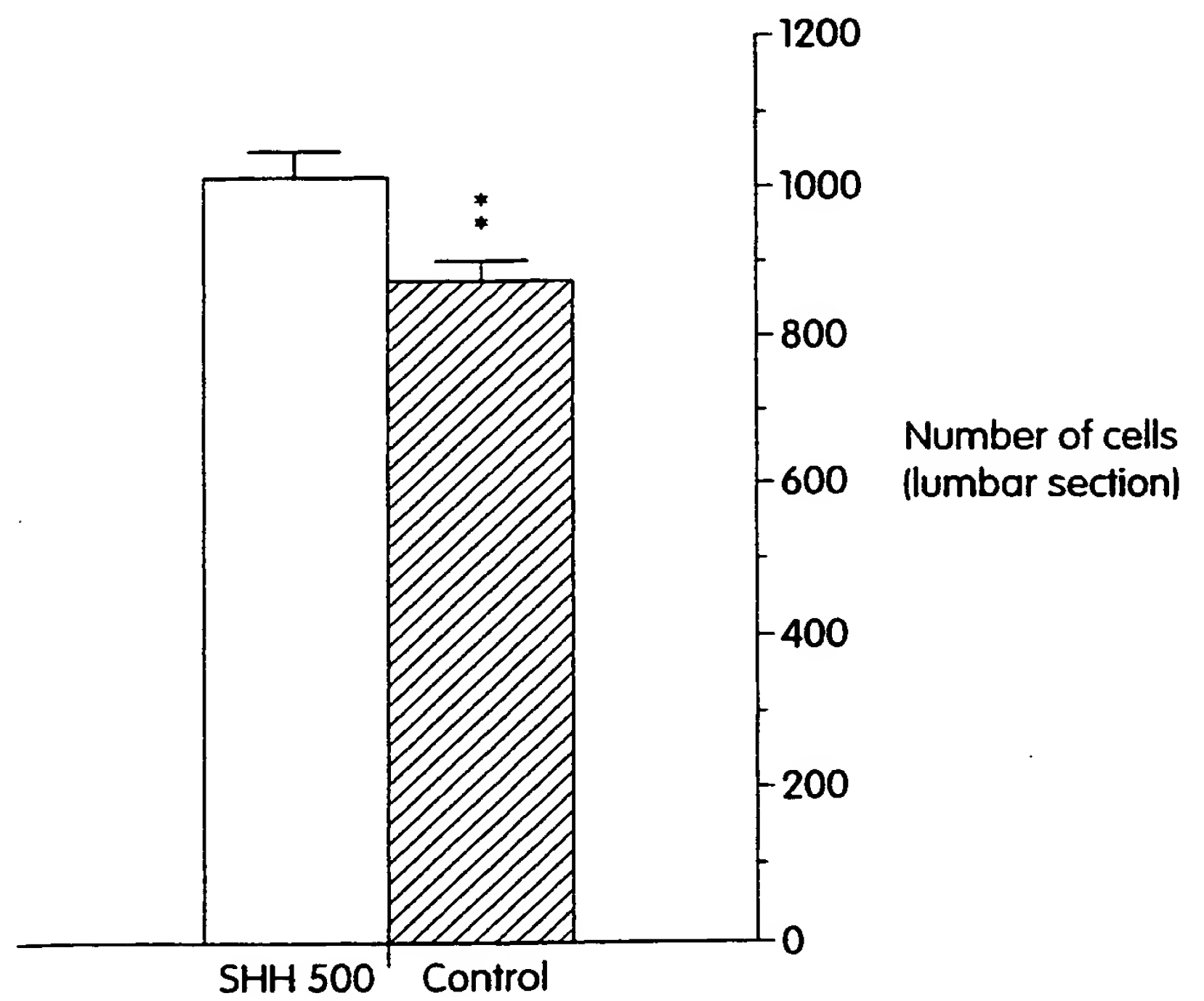


Fig. 19

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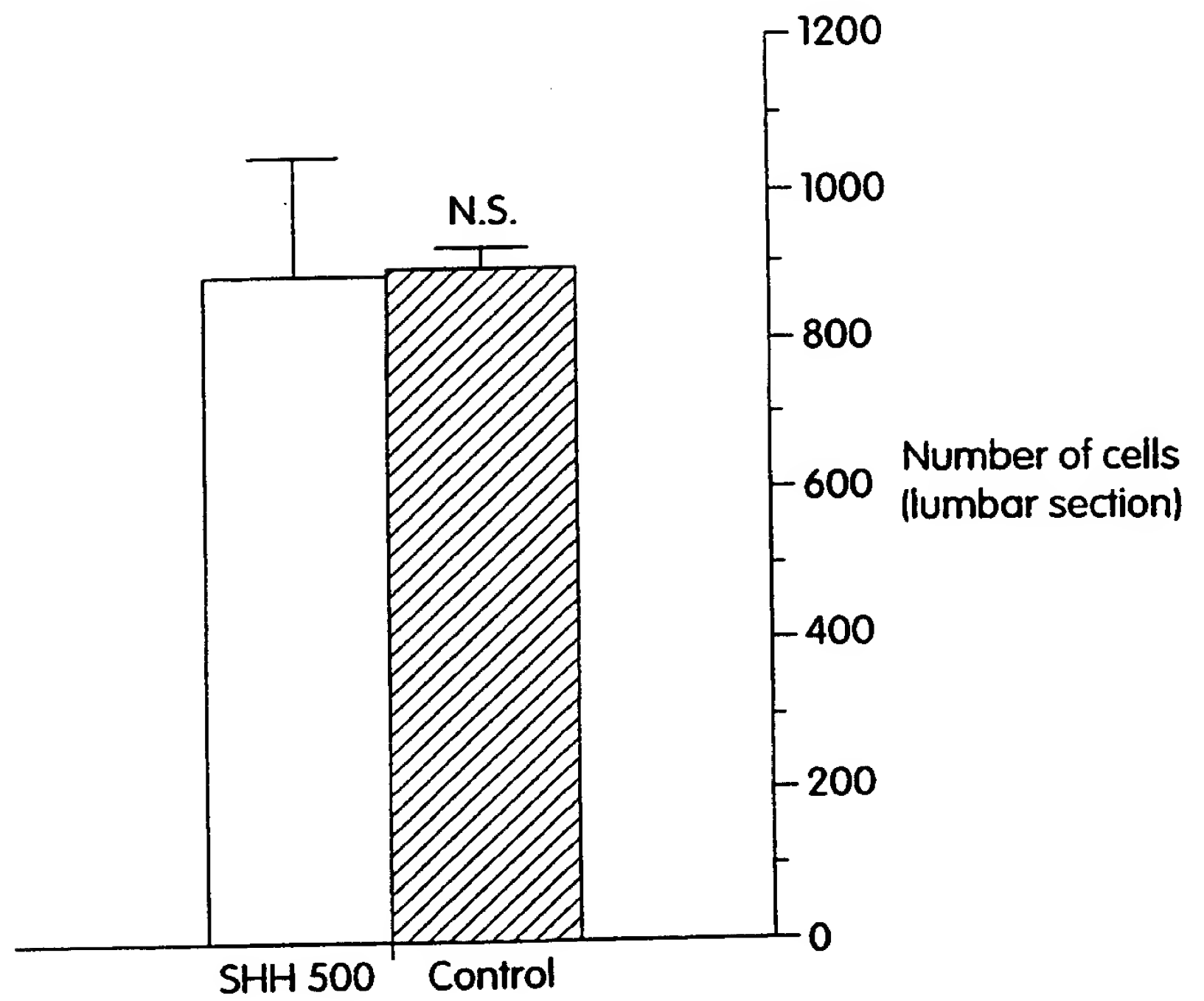


Fig. 20

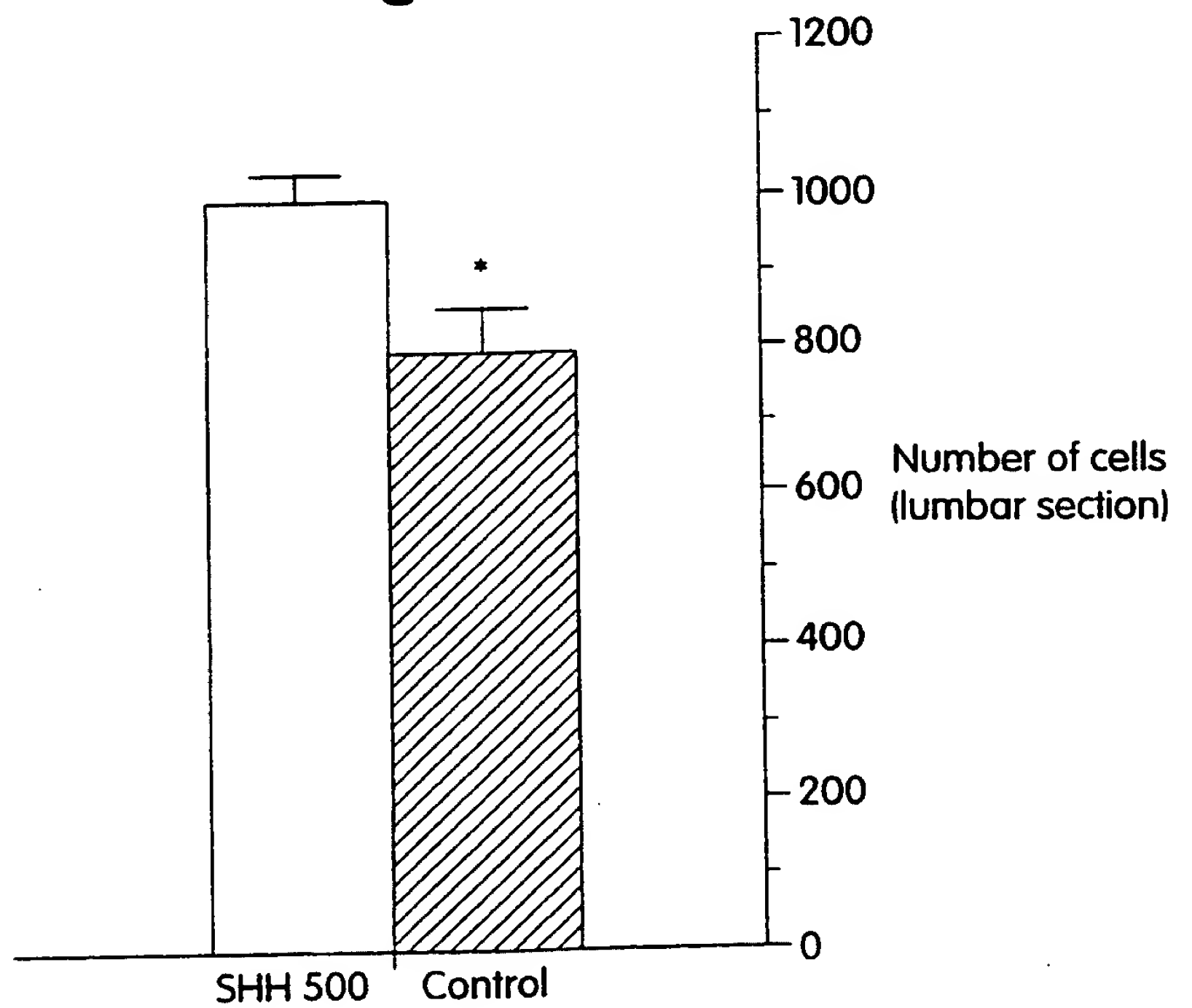


Fig. 21

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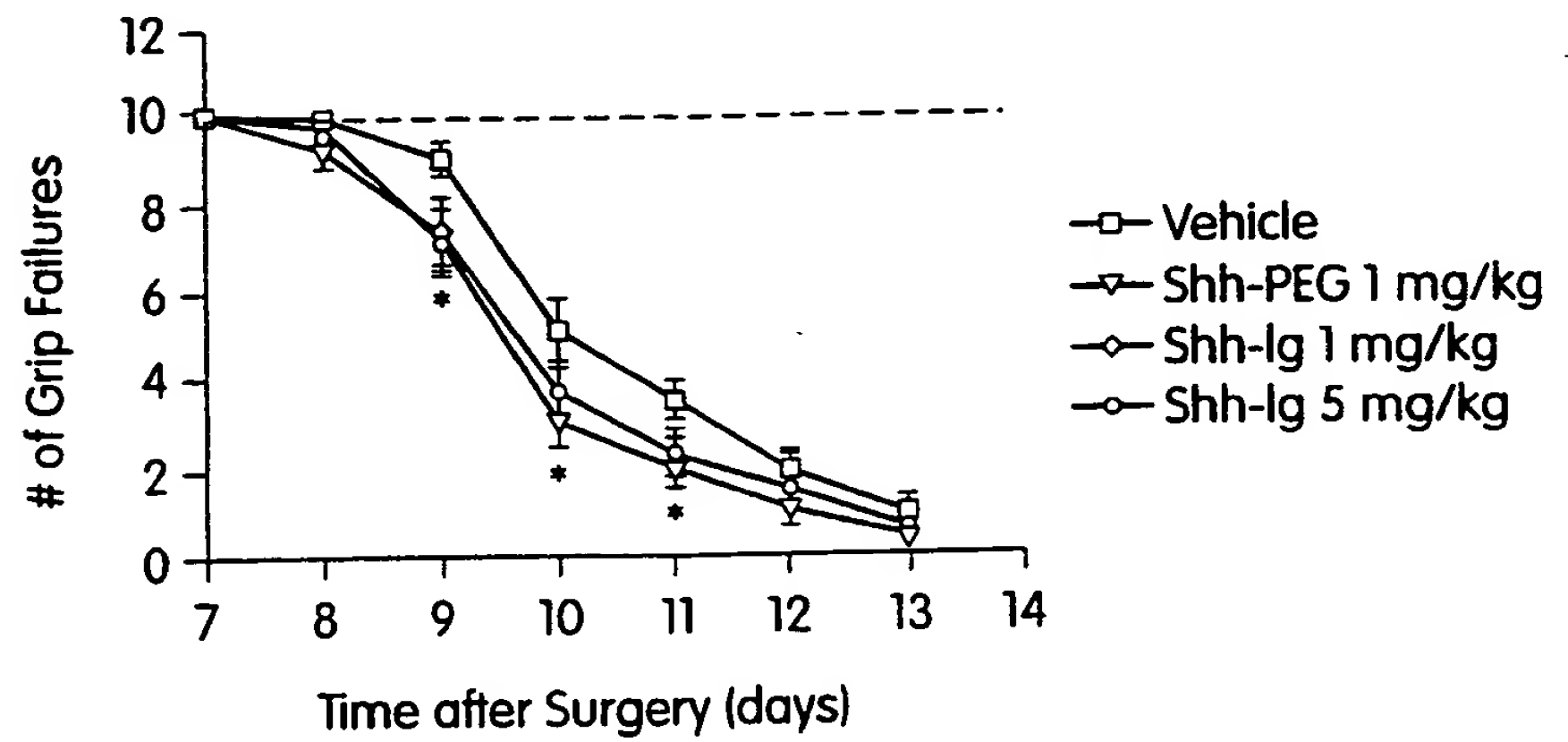


Fig. 22

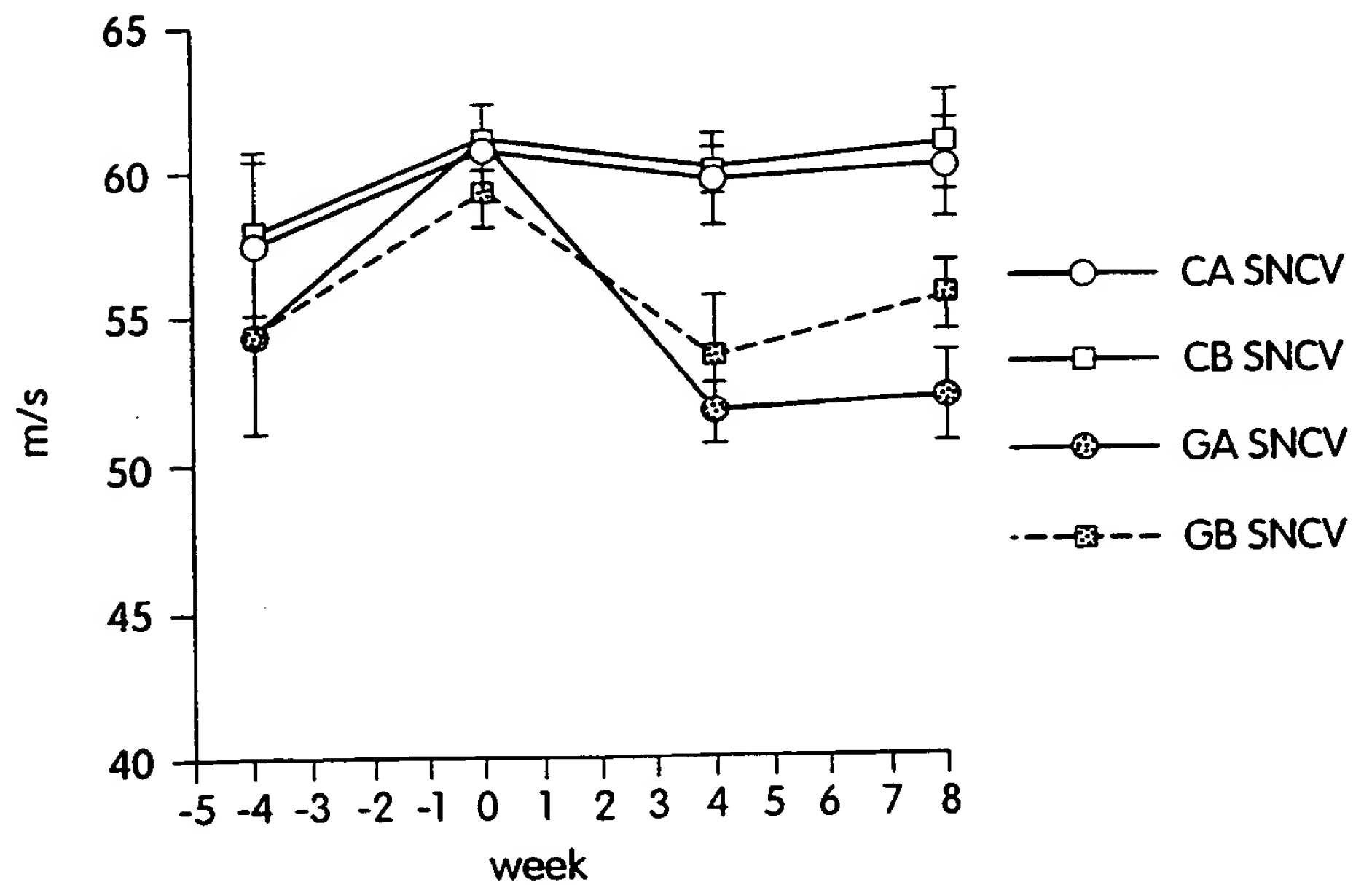


Fig. 23

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SEQUENCE LISTING

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 ONTOGENY, INC..
 GALDES, ALPHONSE
 MAHANTHAPPA, NAGESH

<120> METHODS AND COMPOSITIONS FOR TREATING OR PREVENTING
 PERIPHERAL NEUROPATHIES

<130> BIV-052.25

<140> PCT/US99/26334

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SUBSTITUTE SHEET (RULE 26)

3/44

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SUBSTITUTE SHEET (RULE 26)

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325 330 335	
aac gac gtc ctc gcc tcc tgc tac gcg gtt cta gag agt cac cag tgg	1056
Asn Asp Val Leu Ala Ser Cys Tyr Ala Val Leu Glu Ser His Gln Trp	
340 345 350	

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gcc cac cgc gcc ttc gcc cct ttg cgg ctg ctg cac gcg ctc ggg gct 1104
 Ala His Arg Ala Phe Ala Pro Leu Arg Leu Leu His Ala Leu Gly Ala
 355 360 365

ctg ctc cct ggg ggt gca gtc cag ccg act ggc atg cat tgg tac tct 1152
 Leu Leu Pro Gly Gly Ala Val Gln Pro Thr Gly Met His Trp Tyr Ser
 370 375 380

cgc ctc ctt tac cgc ttg gcc gag gag tta atg ggc tg 1190
 Arg Leu Leu Tyr Arg Leu Ala Glu Glu Leu Met Gly
 385 390 395

<210> 3
 <211> 1281
 <212> DNA
 <213> murine Ihh

<220>
 <221> CDS
 <222> (1) .. (1233)

<400> 3
 atg tct ccc gcc tgg ctc cgg ccc cga ctg cgg ttc tgt ctg ttc ctg 48
 Met Ser Pro Ala Trp Leu Arg Pro Arg Leu Arg Phe Cys Leu Phe Leu
 1 5 10 15

ctg ctg ctg ctt ctg gtg ccg gcg gcg cgg ggc tgc ggg ccg ggc cgg 96
 Leu Leu Leu Leu Leu Val Pro Ala Ala Arg Gly Cys Gly Pro Gly Arg
 20 25 30

gtg gtg ggc agc cgc cgg agg ccg cct cgc aag ctc gtg cct ctt gcc 144
 Val Val Gly Ser Arg Arg Arg Pro Pro Arg Lys Leu Val Pro Leu Ala
 35 40 45

tac aag cag ttc agc ccc aac gtg ccg gag aag acc ctg ggc gcc agc 192
 Tyr Lys Gln Phe Ser Pro Asn Val Pro Glu Lys Thr Leu Gly Ala Ser
 50 55 60

ggg cgc tac gaa ggc aag atc gcg cgc agc tct gag cgc ttc aaa gag 240
 Gly Arg Tyr Glu Gly Lys Ile Ala Arg Ser Ser Glu Arg Phe Lys Glu
 65 70 75 80

ctc acc ccc aac tac aat ccc gac atc atc ttc aag gac gag gag aac 288
 Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn
 85 90 95

acg ggt gcc gac cgc ctc atg acc cag cgc tgc aag gac cgt ctg aac 336
 Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Arg Leu Asn
 100 105 110

tca ctg gcc atc tct gtc atg aac cag tgg cct ggt gtg aaa ctg cgg 384
 Ser Leu Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg
 115 120 125

gtg acc gaa ggc cgg gat gaa gat ggc cat cac tca gag gag tct tta 432
 Val Thr Glu Gly Arg Asp Glu Asp Gly His His Ser Glu Glu Ser Leu
 130 135 140

cac tat gag ggc cgc gcg gtg gat atc acc acc tca gac cgt gac cga 480
 His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg

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145	150	155	160	
aat aag tat gga ctg	ctg gcg cgc tta gca	gtg gag gcc ggc ttc gac		528
Asn Lys Tyr Gly Leu	Leu Ala Arg Leu Ala	Val Glu Ala Gly Phe Asp		
	165	170	175	
tgg gtg tat tac gag	tcc aag gcc cac gtg	cat tgc tct gtc aag tct		576
Trp Val Tyr Tyr Glu	Ser Lys Ala His Val	His Cys Ser Val Lys Ser		
	180	185	190	
gag cat tcg gcc gct gcc	aag aca ggt ggc tgc	ttt cct gcc gga gcc		624
Glu His Ser Ala Ala	Lys Thr Gly Gly Cys	Phe Pro Ala Gly Ala		
	195	200	205	
cag gtg cgc cta gag	aac ggg gag cgt gtg	gcc ctg tca gct gta aag		672
Gln Val Arg Leu Glu	Asn Gly Glu Arg Val	Ala Leu Ser Ala Val Lys		
	210	215	220	
cca gga gac cgg gtg	ctg gcc atg ggg gag	gat ggg acc ccc acc ttc		720
Pro Gly Asp Arg Val	Leu Ala Met Gly Glu	Asp Gly Thr Pro Thr Phe		
	225	230	235	240
agt gat gtg ctt att	ttc ctg gac cgc gag	cca aac cgg ctg aga gct		768
Ser Asp Val Leu Ile	Phe Leu Asp Arg Glu	Pro Asn Arg Leu Arg Ala		
	245	250	255	
ttc cag gtc atc gag	act cag gat cct ccg	cgt cgg ctg gcg ctc acg		816
Phe Gln Val Ile Glu	Thr Gln Asp Pro Pro	Arg Arg Leu Ala Leu Thr		
	260	265	270	
cct gcc cac ctg ctc	ttc att gcg gac aat	cat aca gaa cca gca gcc		864
Pro Ala His Leu Leu	Phe Ile Ala Asp Asn	His Thr Glu Pro Ala Ala		
	275	280	285	
cac ttc cgg gcc aca	ttt gcc agc cat gtg	caa cca ggc caa tat gtg		912
His Phe Arg Ala Thr	Phe Ala Ser His Val	Gln Pro Gly Gln Tyr Val		
	290	295	300	
ctg gta tca ggg gta	cca ggc ctc cag cct	gct cgg gtg gca gct gtc		960
Leu Val Ser Gly Val	Pro Gly Leu Gln Pro	Ala Arg Val Ala Ala Val		
	305	310	315	320
tcc acc cac gtg gcc	ctt ggg tcc tat gct	cct ctc aca agg cat ggg		1008
Ser Thr His Val Ala	Leu Gly Ser Tyr Ala	Pro Leu Thr Arg His Gly		
	325	330	335	
aca ctt gtg gtg gag	gat gtg gtg gcc tcc	tgc ttt gca gct gtg gct		1056
Thr Leu Val Val Glu	Asp Val Val Ala Ser	Cys Phe Ala Ala Val Ala		
	340	345	350	
gac cac cat ctg gct	cag ttg gcc ttc tgg	ccc ctg cga ctg ttt ccc		1104
Asp His His Leu Ala	Gln Leu Ala Phe Trp	Pro Leu Arg Leu Phe Pro		
	355	360	365	
agt ttg gca tgg ggc	agc tgg acc cca agt	gag ggt gtt cac tcc tac		1152
Ser Leu Ala Trp Gly	Ser Trp Thr Pro Ser	Glu Gly Val His Ser Tyr		
	370	375	380	
cct cag atg ctc tac	cgc ctg ggg cgt ctc	ttg cta gaa gag agc acc		1200
Pro Gln Met Leu Tyr	Arg Leu Gly Arg Leu	Leu Leu Glu Glu Ser Thr		

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385											390											395											400
ttc cat cca ctg ggc atg tct ggg gca gga agc tgaagggact ctaaccactg																				1253													
Phe His Pro Leu Gly Met Ser Gly Ala Gly Ser																																	
										405											410												
ccctcctgga actgctgtgc gtggatcc																				1281													
<210> 4																																	
<211> 1313																																	
<212> DNA																																	
<213> murine Shh																																	
<220>																																	
<221> CDS																																	
<222> (1)..(1311)																																	
<400> 4																																	
atg	ctg	ctg	ctg	ctg	gcc	aga	tgt	ttt	ctg	gtg	atc	ctt	gct	tcc	tcg					48													
Met	Leu	Leu	Leu	Leu	Ala	Arg	Cys	Phe	Leu	Val	Ile	Leu	Ala	Ser	Ser																		
1					5					10					15																		
ctg	ctg	gtg	tgc	ccc	ggg	ctg	gcc	tgt	ggg	ccc	ggc	agg	ggg	ttt	gga					96													
Leu	Leu	Val	Cys	Pro	Gly	Leu	Ala	Cys	Gly	Pro	Gly	Arg	Gly	Phe	Gly																		
			20				25				30																						
aag	agg	cgg	cac	ccc	aaa	aag	ctg	acc	cct	tta	gcc	tac	aag	cag	ttt					144													
Lys	Arg	Arg	His	Pro	Lys	Lys	Leu	Thr	Pro	Leu	Ala	Tyr	Lys	Gln	Phe																		
		35			40			45																									
att	ccc	aac	gta	gcc	gag	aag	acc	cta	ggg	gcc	agc	ggc	aga	tat	gaa					192													
Ile	Pro	Asn	Val	Ala	Glu	Lys	Thr	Leu	Gly	Ala	Ser	Gly	Arg	Tyr	Glu																		
		50			55			60																									
ggg	aag	atc	aca	aga	aac	tcc	gaa	cga	ttt	aag	gaa	ctc	acc	ccc	aat					240													
Gly	Lys	Ile	Thr	Arg	Asn	Ser	Glu	Arg	Phe	Lys	Glu	Leu	Thr	Pro	Asn																		
65					70					75					80																		
tac	aac	ccc	gac	atc	ata	ttt	aag	gat	gag	gaa	aac	acg	gga	gca	gac					288													
Tyr	Asn	Pro	Asp	Ile	Ile	Phe	Lys	Asp	Glu	Glu	Asn	Thr	Gly	Ala	Asp																		
				85					90					95																			
cgg	ctg	atg	act	cag	agg	tgc	aaa	gac	aag	tta	aat	gcc	ttg	gcc	atc					336													
Arg	Leu	Met	Thr	Gln	Arg	Cys	Lys	Asp	Lys	Leu	Asn	Ala	Leu	Ala	Ile																		
			100				105				110																						
tct	gtg	atg	aac	cag	tgg	cct	gga	gtg	agg	ctg	cga	gtg	acc	gag	ggc					384													
Ser	Val	Met	Asn	Gln	Trp	Pro	Gly	Val	Arg	Leu	Arg	Val	Thr	Glu	Gly																		
		115			120			125																									
tgg	gat	gag	gac	ggc	cat	cat	tca	gag	gag	tct	cta	cac	tat	gag	ggc					432													
Trp	Asp	Glu	Asp	Gly	His	His	Ser	Glu	Glu	Ser	Leu	His	Tyr	Glu	Gly																		
		130			135			140																									
cga	gca	gtg	gac	atc	acc	acg	tcc	gac	cgg	gac	cgc	agc	aag	tac	ggc					480													
Arg	Ala	Val	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Arg	Ser	Lys	Tyr	Gly																		
145					150					155					160																		
atg	ctg	gct	cgc	ctg	gct	gtg	gaa	gca	ggt	ttc	gac	tgg	gtc	tac	tat					528													

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Met	Leu	Ala	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	Tyr	Tyr	
				165					170					175		
gaa	tcc	aaa	gct	cac	atc	cac	tgt	tct	gtg	aaa	gca	gag	aac	tcc	gtg	576
Glu	Ser	Lys	Ala	His	Ile	His	Cys	Ser	Val	Lys	Ala	Glu	Asn	Ser	Val	
			180				185						190			
gcg	gcc	aaa	tcc	ggc	ggc	tgt	ttc	ccg	gga	tcc	gcc	acc	gtg	cac	ctg	624
Ala	Ala	Lys	Ser	Gly	Gly	Cys	Phe	Pro	Gly	Ser	Ala	Thr	Val	His	Leu	
		195				200					205					
gag	cag	ggc	ggc	acc	aag	ctg	gtg	aag	gac	tta	cgt	ccc	gga	gac	cgc	672
Glu	Gln	Gly	Gly	Thr	Lys	Leu	Val	Lys	Asp	Leu	Arg	Pro	Gly	Asp	Arg	
	210					215					220					
gtg	ctg	gcg	gct	gac	gac	cag	ggc	cgg	ctg	ctg	tac	agc	gac	ttc	ctc	720
Val	Leu	Ala	Ala	Asp	Asp	Gln	Gly	Arg	Leu	Leu	Tyr	Ser	Asp	Phe	Leu	
	225				230					235					240	
acc	ttc	ctg	gac	cgc	gac	gaa	ggc	gcc	aag	aag	gtc	ttc	tac	gtg	atc	768
Thr	Phe	Leu	Asp	Arg	Asp	Glu	Gly	Ala	Lys	Lys	Val	Phe	Tyr	Val	Ile	
				245					250					255		
gag	acg	ctg	gag	ccg	cgc	gag	cgc	ctg	ctg	ctc	acc	gcc	gcg	cac	ctg	816
Glu	Thr	Leu	Glu	Pro	Arg	Glu	Arg	Leu	Leu	Leu	Thr	Ala	Ala	His	Leu	
			260					265					270			
ctc	ttc	gtg	gcg	ccg	cac	aac	gac	tcg	ggg	ccc	acg	ccc	ggg	cca	agc	864
Leu	Phe	Val	Ala	Pro	His	Asn	Asp	Ser	Gly	Pro	Thr	Pro	Gly	Pro	Ser	
		275					280					285				
gcg	ctc	ttt	gcc	agc	cgc	gtg	cgc	ccc	ggg	cag	cgc	gtg	tac	gtg	gtg	912
Ala	Leu	Phe	Ala	Ser	Arg	Val	Arg	Pro	Gly	Gln	Arg	Val	Tyr	Val	Val	
	290					295					300					
gct	gaa	cgc	ggc	ggg	gac	cgc	cgg	ctg	ctg	ccc	gcc	gcg	gtg	cac	agc	960
Ala	Glu	Arg	Gly	Gly	Asp	Arg	Arg	Leu	Leu	Pro	Ala	Ala	Val	His	Ser	
	305				310					315					320	
gtg	acg	ctg	cga	gag	gag	gag	gcg	ggc	gcg	tac	gcg	ccg	ctc	acg	gcg	1008
Val	Thr	Leu	Arg	Glu	Glu	Glu	Ala	Gly	Ala	Tyr	Ala	Pro	Leu	Thr	Ala	
				325				330						335		
cac	ggc	acc	att	ctc	atc	aac	cgg	gtg	ctc	gcc	tcg	tgc	tac	gct	gtc	1056
His	Gly	Thr	Ile	Leu	Ile	Asn	Arg	Val	Leu	Ala	Ser	Cys	Tyr	Ala	Val	
			340					345					350			
atc	gag	gag	cac	agc	tgg	gca	cac	cgg	gcc	ttc	gcg	cct	ttc	cgc	ctg	1104
Ile	Glu	Glu	His	Ser	Trp	Ala	His	Arg	Ala	Phe	Ala	Pro	Phe	Arg	Leu	
		355					360					365				
gcg	cac	gcg	ctg	ctg	gcc	gcg	ctg	gca	ccc	gcc	cgc	acg	gac	ggc	ggg	1152
Ala	His	Ala	Leu	Leu	Ala	Ala	Leu	Ala	Pro	Ala	Arg	Thr	Asp	Gly	Gly	
		370				375					380					
ggc	ggg	ggc	agc	atc	cct	gca	gcg	caa	tct	gca	acg	gaa	gcg	agg	ggc	1200
Gly	Gly	Gly	Ser	Ile	Pro	Ala	Ala	Gln	Ser	Ala	Thr	Glu	Ala	Arg	Gly	
	385				390				395						400	
gcg	gag	ccg	act	gcg	ggc	atc	cac	tgg	tac	tcg	cag	ctg	ctc	tac	cac	1248

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Ala	Glu	Pro	Thr	Ala	Gly	Ile	His	Trp	Tyr	Ser	Gln	Leu	Leu	Tyr	His	
				405					410					415		
att	ggc	acc	tgg	ctg	ttg	gac	agc	gag	acc	atg	cat	ccc	ttg	gga	atg	1296
Ile	Gly	Thr	Trp	Leu	Leu	Asp	Ser	Glu	Thr	Met	His	Pro	Leu	Gly	Met	
			420					425					430			
gcg	gtc	aag	tcc	agc	tg											1313
Ala	Val	Lys	Ser	Ser												
			435													
<210> 5																
<211> 1256																
<212> DNA																
<213> zebrafish Shh																
<220>																
<221> CDS																
<222> (1) .. (1254)																
<400> 5																
atg	cgg	ctt	ttg	acg	aga	gtg	ctg	ctg	gtg	tct	ctt	ctc	act	ctg	tcc	48
Met	Arg	Leu	Leu	Thr	Arg	Val	Leu	Leu	Val	Ser	Leu	Leu	Thr	Leu	Ser	
1				5					10					15		
ttg	gtg	gtg	tcc	gga	ctg	gcc	tgc	ggc	cct	ggc	aga	ggc	tac	ggc	aga	96
Leu	Val	Val	Ser	Gly	Leu	Ala	Cys	Gly	Pro	Gly	Arg	Gly	Tyr	Gly	Arg	
			20					25					30			
aga	aga	cat	ccg	aag	aag	ctg	aca	cct	ctc	gcc	tac	aag	cag	ttc	ata	144
Arg	Arg	His	Pro	Lys	Lys	Leu	Thr	Pro	Leu	Ala	Tyr	Lys	Gln	Phe	Ile	
			35				40					45				
cct	aat	gtc	gcg	gag	aag	acc	tta	ggg	gcc	agc	ggc	aga	tac	gag	ggc	192
Pro	Asn	Val	Ala	Glu	Lys	Thr	Leu	Gly	Ala	Ser	Gly	Arg	Tyr	Glu	Gly	
	50					55					60					
aag	ata	acg	cgc	aat	tcg	gag	aga	ttt	aaa	gaa	ctt	act	cca	aat	tac	240
Lys	Ile	Thr	Arg	Asn	Ser	Glu	Arg	Phe	Lys	Glu	Leu	Thr	Pro	Asn	Tyr	
65					70				75						80	
aat	ccc	gac	att	atc	ttt	aag	gat	gag	gag	aac	acg	gga	gcg	gac	agg	288
Asn	Pro	Asp	Ile	Ile	Phe	Lys	Asp	Glu	Glu	Asn	Thr	Gly	Ala	Asp	Arg	
			85					90						95		
ctc	atg	aca	cag	aga	tgc	aaa	gac	aag	ctg	aac	tcg	ctg	gcc	atc	tct	336
Leu	Met	Thr	Gln	Arg	Cys	Lys	Asp	Lys	Leu	Asn	Ser	Leu	Ala	Ile	Ser	
			100					105					110			
gta	atg	aac	cac	tgg	cca	ggg	gtt	aag	ctg	cgt	gtg	aca	gag	ggc	tgg	384
Val	Met	Asn	His	Trp	Pro	Gly	Val	Lys	Leu	Arg	Val	Thr	Glu	Gly	Trp	
			115				120					125				
gat	gag	gac	ggc	cac	cat	ttt	gaa	gaa	tca	ctc	cac	tac	gag	gga	aga	432
Asp	Glu	Asp	Gly	His	His	Phe	Glu	Glu	Ser	Leu	His	Tyr	Glu	Gly	Arg	
			130				135				140					
gct	gtt	gat	att	acc	acc	tct	gac	cga	gac	aag	agc	aaa	tac	ggg	aca	480
Ala	Val	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Lys	Ser	Lys	Tyr	Gly	Thr	
145					150					155					160	

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ctg tct cgc cta gct gtg gag gct gga ttt gac tgg gtc tat tac gag	528
Leu Ser Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu	
165 170 175	
tcc aaa gcc cac att cat tgc tct gtc aaa gca gaa aat tcg gtt gct	576
Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val Ala	
180 185 190	
gcg aaa tct ggg ggc tgt ttc cca ggt tcg gct ctg gtc tcg ctc cag	624
Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Leu Val Ser Leu Gln	
195 200 205	
gac gga gga cag aag gcc gtg aag gac ctg aac ccc gga gac aag gtg	672
Asp Gly Gly Gln Lys Ala Val Lys Asp Leu Asn Pro Gly Asp Lys Val	
210 215 220	
ctg gcg gca gac agc gcg gga aac ctg gtg ttc agc gac ttc atc atg	720
Leu Ala Ala Asp Ser Ala Gly Asn Leu Val Phe Ser Asp Phe Ile Met	
225 230 235 240	
ttc aca gac cga gac tcc acg acg cga cgt gtg ttt tac gtc ata gaa	768
Phe Thr Asp Arg Asp Ser Thr Thr Arg Arg Val Phe Tyr Val Ile Glu	
245 250 255	
acg caa gaa ccc gtt gaa aag atc acc ctc acc gcc gct cac ctc ctt	816
Thr Gln Glu Pro Val Glu Lys Ile Thr Leu Thr Ala Ala His Leu Leu	
260 265 270	
ttt gtc ctc gac aac tca acg gaa gat ctc cac acc atg acc gcc gcg	864
Phe Val Leu Asp Asn Ser Thr Glu Asp Leu His Thr Met Thr Ala Ala	
275 280 285	
tat gcc agc agt gtc aga gcc gga caa aag gtg atg gtt gtt gat gat	912
Tyr Ala Ser Ser Val Arg Ala Gly Gln Lys Val Met Val Val Asp Asp	
290 295 300	
agc ggt cag ctt aaa tct gtc atc gtg cag cgg ata tac acg gag gag	960
Ser Gly Gln Leu Lys Ser Val Ile Val Gln Arg Ile Tyr Thr Glu Glu	
305 310 315 320	
cag cgg ggc tcg ttc gca cca gtg act gca cat ggg acc att gtg gtc	1008
Gln Arg Gly Ser Phe Ala Pro Val Thr Ala His Gly Thr Ile Val Val	
325 330 335	
gac aga ata ctg gcg tcc tgt tac gcc gta ata gag gac cag ggg ctt	1056
Asp Arg Ile Leu Ala Ser Cys Tyr Ala Val Ile Glu Asp Gln Gly Leu	
340 345 350	
gcg cat ttg gcc ttc gcg ccc gcc agg ctc tat tat tac gtg tca tca	1104
Ala His Leu Ala Phe Ala Pro Ala Arg Leu Tyr Tyr Tyr Val Ser Ser	
355 360 365	
ttc ctg tcc ccc aaa act cca gca gtc ggt cca atg cga ctt tac aac	1152
Phe Leu Ser Pro Lys Thr Pro Ala Val Gly Pro Met Arg Leu Tyr Asn	
370 375 380	
agg agg ggg tcc act ggt act cca ggc tcc tgt cat caa atg gga acg	1200
Arg Arg Gly Ser Thr Gly Thr Pro Gly Ser Cys His Gln Met Gly Thr	
385 390 395 400	

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tgg ctt ttg gac agc aac atg ctt cat cct ttg ggg atg tca gta aac 1248
 Trp Leu Leu Asp Ser Asn Met Leu His Pro Leu Gly Met Ser Val Asn
 405 410 415

tca agc tg 1256
 Ser Ser

<210> 6
 <211> 1425
 <212> DNA
 <213> Homo sapien Shh

<220>
 <221> CDS
 <222> (1)..(1425)

<220>
 <223> "nnn" encoding "Xaa" at position 1387-1389 may be a, t, c,
 g, other or unknown

<400> 6
 atg ctg ctg ctg gcg aga tgt ctg ctg cta gtc ctc gtc tcc tcg ctg 48
 Met Leu Leu Leu Ala Arg Cys Leu Leu Leu Val Leu Val Ser Ser Leu
 1 5 10 15

ctg gta tgc tcg gga ctg gcg tgc gga ccg ggc agg ggg ttc ggg aag 96
 Leu Val Cys Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly Lys
 20 25 30

agg agg cac ccc aaa aag ctg acc cct tta gcc tac aag cag ttt atc 144
 Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile
 35 40 45

ccc aat gtg gcc gag aag acc cta ggc gcc agc gga agg tat gaa ggg 192
 Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly
 50 55 60

aag atc tcc aga aac tcc gag cga ttt aag gaa ctc acc ccc aat tac 240
 Lys Ile Ser Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr
 65 70 75 80

aac ccc gac atc ata ttt aag gat gaa gaa aac acc gga gcg gac agg 288
 Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp Arg
 85 90 95

ctg atg act cag agg tgt aag gac aag ttg aac gct ttg gcc atc tcg 336
 Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile Ser
 100 105 110

gtg atg aac cag tgg cca gga gtg aaa ctg cgg gtg acc gag ggc tgg 384
 Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp
 115 120 125

gac gaa gat ggc cac cac tca gag gag tct ctg cac tac gag ggc cgc 432
 Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly Arg
 130 135 140

gca gtg gac atc acc acg tct gac cgc gac cgc agc aag tac ggc atg 480
 Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly Met
 145 150 155 160

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ctg gcc cgc ctg gcg gtg gag gcc ggc ttc gac tgg gtg tac tac gag	528
Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu	
165 170 175	
tcc aag gca cat atc cac tgc tcg gtg aaa gca gag aac tcg gtg gcg	576
Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val Ala	
180 185 190	
gcc aaa tcg gga ggc tgc ttc ccg ggc tcg gcc acg gtg cac ctg gag	624
Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val His Leu Glu	
195 200 205	
cag ggc ggc acc aag ctg gtg aag gac ctg agc ccc ggg gac cgc gtg	672
Gln Gly Gly Thr Lys Leu Val Lys Asp Leu Ser Pro Gly Asp Arg Val	
210 215 220	
ctg gcg gcg gac gac cag ggc cgg ctg ctc tac agc gac ttc ctc act	720
Leu Ala Ala Asp Asp Gln Gly Arg Leu Leu Tyr Ser Asp Phe Leu Thr	
225 230 235 240	
ttc ctg gac cgc gac gac ggc gcc aag aag gtc ttc tac gtg atc gag	768
Phe Leu Asp Arg Asp Asp Gly Ala Lys Lys Val Phe Tyr Val Ile Glu	
245 250 255	
acg cgg gag ccg cgc gag cgc ctg ctg ctc acc gcc gcg cac ctg ctc	816
Thr Arg Glu Pro Arg Glu Arg Leu Leu Leu Thr Ala Ala His Leu Leu	
260 265 270	
ttt gtg gcg ccg cac aac gac tcg gcc acc ggg gag ccc gag gcg tcc	864
Phe Val Ala Pro His Asn Asp Ser Ala Thr Gly Glu Pro Glu Ala Ser	
275 280 285	
tcg ggc tcg ggg ccg cct tcc ggg ggc gca ctg ggg cct cgg gcg ctg	912
Ser Gly Ser Gly Pro Pro Ser Gly Gly Ala Leu Gly Pro Arg Ala Leu	
290 295 300	
ttc gcc agc cgc gtg cgc ccg ggc cag cgc gtg tac gtg gtg gcc gag	960
Phe Ala Ser Arg Val Arg Pro Gly Gln Arg Val Tyr Val Val Ala Glu	
305 310 315 320	
cgt gac ggg gac cgc cgg ctc ctg ccc gcc gct gtg cac agc gtg acc	1008
Arg Asp Gly Asp Arg Arg Leu Leu Pro Ala Ala Val His Ser Val Thr	
325 330 335	
cta agc gag gag gcc gcg ggc gcc tac gcg ccg ctc acg gcc cag ggc	1056
Leu Ser Glu Glu Ala Ala Gly Ala Tyr Ala Pro Leu Thr Ala Gln Gly	
340 345 350	
acc att ctc atc aac cgg gtg ctg gcc tcg tgc tac gcg gtc atc gag	1104
Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val Ile Glu	
355 360 365	
gag cac agc tgg gcg cac cgg gcc ttc gcg ccc ttc cgc ctg gcg cac	1152
Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu Ala His	
370 375 380	
gcg ctc ctg gct gca ctg gcg ccc gcg cgc acg gac cgc ggc ggg gac	1200
Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Arg Gly Gly Asp	
385 390 395 400	

SUBSTITUTE SHEET (RULE 26)

13/44

agc ggc ggc ggg gac cgc ggg ggc ggc ggc ggc aga gta gcc cta acc 1248
 Ser Gly Gly Gly Asp Arg Gly Gly Gly Gly Gly Arg Val Ala Leu Thr
 405 410 415

gct cca ggt gct gcc gac gct ccg ggt gcg ggg gcc acc gcg ggc atc 1296
 Ala Pro Gly Ala Ala Asp Ala Pro Gly Ala Gly Ala Thr Ala Gly Ile
 420 425 430

cac tgg tac tcg cag ctg ctc tac caa ata ggc acc tgg ctc ctg gac 1344
 His Trp Tyr Ser Gln Leu Leu Tyr Gln Ile Gly Thr Trp Leu Leu Asp
 435 440 445

agc gag gcc ctg cac ccg ctg ggc atg gcg gtc aag tcc agc nnn agc 1392
 Ser Glu Ala Leu His Pro Leu Gly Met Ala Val Lys Ser Ser Xaa Ser
 450 455 460

cgg ggg gcc ggg gga ggg gcg cgg gag ggg gcc 1425
 Arg Gly Ala Gly Gly Gly Ala Arg Glu Gly Ala
 465 470 475

<210> 7
 <211> 1622
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 <213> Homo sapien Ihh

<220>
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 <222> (51) .. (1283)

<400> 7
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 Met Ser
 1

ccc gcc cgg ctc cgg ccc cga ctg cac ttc tgc ctg gtc ctg ttg ctg 104
 Pro Ala Arg Leu Arg Pro Arg Leu His Phe Cys Leu Val Leu Leu Leu
 5 10 15

ctg ctg gtg gtg ccc gcg gca tgg ggc tgc ggg ccg ggt cgg gtg gtg 152
 Leu Leu Val Val Pro Ala Ala Trp Gly Cys Gly Pro Gly Arg Val Val
 20 25 30

ggc agc cgc cgg cga ccg cca cgc aaa ctc gtg ccg ctc gcc tac aag 200
 Gly Ser Arg Arg Arg Pro Pro Arg Lys Leu Val Pro Leu Ala Tyr Lys
 35 40 45 50

cag ttc agc ccc aat gtg ccc gag aag acc ctg ggc gcc agc gga cgc 248
 Gln Phe Ser Pro Asn Val Pro Glu Lys Thr Leu Gly Ala Ser Gly Arg
 55 60 65

tat gaa ggc aag atc gct cgc agc tcc gag cgc ttc aag gag ctc acc 296
 Tyr Glu Gly Lys Ile Ala Arg Ser Ser Glu Arg Phe Lys Glu Leu Thr
 70 75 80

ccc aat tac aat cca gac atc atc ttc aag gac gag gag aac aca ggc 344
 Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly
 85 90 95

gcc gac cgc ctc atg acc cag cgc tgc aag gac cgc ctg aac tcg ctg 392
 Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Arg Leu Asn Ser Leu

SUBSTITUTE SHEET (RULE 26)

14/44

100	105	110	
gct atc tcg gtg atg aac cag tgg ccc ggt gtg aag ctg cgg gtg acc Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr 115 120 125 130			440
gag ggc tgg gac gag gac ggc cac cac tca gag gag tcc ctg cat tat Glu Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr 135 140 145			488
gag ggc cgc gcg gtg gac atc acc aca tca gac cgc gac cgc aat aag Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys 150 155 160			536
tat gga ctg ctg gcg cgc ttg gca gtg gag gcc ggc ttt gac tgg gtg Tyr Gly Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val 165 170 175			584
tat tac gag tca aag gcc cac gtg cat tgc tcc gtc aag tcc gag cac Tyr Tyr Glu Ser Lys Ala His Val His Cys Ser Val Lys Ser Glu His 180 185 190			632
tcg gcc gca gcc aag acg ggc ggc tgc ttc cct gcc gga gcc cag gta Ser Ala Ala Ala Lys Thr Gly Gly Cys Phe Pro Ala Gly Ala Gln Val 195 200 205 210			680
cgc ctg gag agt ggg gcg cgt gtg gcc ttg tca gcc gtg agg ccg gga Arg Leu Glu Ser Gly Ala Arg Val Ala Leu Ser Ala Val Arg Pro Gly 215 220 225			728
gac cgt gtg ctg gcc atg ggg gag gat ggg agc ccc acc ttc agc gat Asp Arg Val Leu Ala Met Gly Glu Asp Gly Ser Pro Thr Phe Ser Asp 230 235 240			776
gtg ctc att ttc ctg gac cgc gag ccc cac agg ctg aga gcc ttc cag Val Leu Ile Phe Leu Asp Arg Glu Pro His Arg Leu Arg Ala Phe Gln 245 250 255			824
gtc atc gag act cag gac ccc cca cgc cgc ctg gca ctc aca ccc gct Val Ile Glu Thr Gln Asp Pro Pro Arg Arg Leu Ala Leu Thr Pro Ala 260 265 270			872
cac ctg ctc ttt acg gct gac aat cac acg gag ccg gca gcc cgc ttc His Leu Leu Phe Thr Ala Asp Asn His Thr Glu Pro Ala Ala Arg Phe 275 280 285 290			920
cgg gcc aca ttt gcc agc cac gtg cag cct ggc cag tac gtg ctg gtg Arg Ala Thr Phe Ala Ser His Val Gln Pro Gly Gln Tyr Val Leu Val 295 300 305			968
gct ggg gtg cca ggc ctg cag cct gcc cgc gtg gca gct gtc tct aca Ala Gly Val Pro Gly Leu Gln Pro Ala Arg Val Ala Ala Val Ser Thr 310 315 320			1016
cac gtg gcc ctc ggg gcc tac gcc ccg ctc aca aag cat ggg aca ctg His Val Ala Leu Gly Ala Tyr Ala Pro Leu Thr Lys His Gly Thr Leu 325 330 335			1064
gtg gtg gag gat gtg gtg gca tcc tgc ttc gcg gcc gtg gct gac cac Val Val Glu Asp Val Val Ala Ser Cys Phe Ala Ala Val Ala Asp His 1112			

SUBSTITUTE SHEET (RULE 26)

15/44

340	345	350	
cac ctg gct cag ttg gcc ttc tgg ccc ctg aga ctc ttt cac agc ttg			1160
His Leu Ala Gln Leu Ala Phe Trp Pro Leu Arg Leu Phe His Ser Leu			
355	360	365	370
gca tgg ggc agc tgg acc ccg ggg gag ggt gtg cat tgg tac ccc cag			1208
Ala Trp Gly Ser Trp Thr Pro Gly Glu Gly Val His Trp Tyr Pro Gln			
	375	380	385
ctg ctc tac cgc ctg ggg cgt ctc ctg cta gaa gag ggc agc ttc cac			1256
Leu Leu Tyr Arg Leu Gly Arg Leu Leu Leu Glu Glu Gly Ser Phe His			
	390	395	400
cca ctg ggc atg tcc ggg gca ggg agc tgaaaggact ccaccgctgc			1303
Pro Leu Gly Met Ser Gly Ala Gly Ser			
	405	410	
cctcctggaa ctgctgtact ggggtccagaa gcctctcagc caggagggag ctggccctgg			1363
aagggacctg agctggggga cactggctcc tgccatctcc tctgccatga agatacacca			1423
ttgagacttg actgggcaac accagcgtcc cccacccgcg tcgtggtgta gtcatagagc			1483
tgcaagctga gctggcgagg ggatggttgt tgaccctct ctctagaga ccttgaggct			1543
ggcacggcga ctcccaactc agcctgctct cactacgagt ttccatactc tgcctcccc			1603
attgggaggg cccattccc			1622
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<211> 1190			
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<213> Homo sapien Dhh			
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<221> CDS			
<222> (1)..(1188)			
<400> 8			
atg gct ctc ctg acc aat cta ctg ccc ttg tgc tgc ttg gca ctt ctg			48
Met Ala Leu Leu Thr Asn Leu Leu Pro Leu Cys Cys Leu Ala Leu Leu			
1	5	10	15
gcg ctg cca gcc cag agc tgc ggg ccg ggc cgg ggg ccg gtt ggc cgg			96
Ala Leu Pro Ala Gln Ser Cys Gly Pro Gly Arg Gly Pro Val Gly Arg			
	20	25	30
cgc cgc tat gcg cgc aag cag ctc gtg ccg cta ctc tac aag caa ttt			144
Arg Arg Tyr Ala Arg Lys Gln Leu Val Pro Leu Leu Tyr Lys Gln Phe			
	35	40	45
gtg ccc ggc gtg cca gag cgg acc ctg ggc gcc agt ggg cca gcg gag			192
Val Pro Gly Val Pro Glu Arg Thr Leu Gly Ala Ser Gly Pro Ala Glu			
	50	55	60
ggg agg gtg gca agg ggc tcc gag cgc ttc cgg gac ctc gtg ccc aac			240
Gly Arg Val Ala Arg Gly Ser Glu Arg Phe Arg Asp Leu Val Pro Asn			
65	70	75	80

SUBSTITUTE SHEET (RULE 26)

16/44

tac aac ccc gac atc atc ttc aag gat gag gag aac agt gga gcc gac	288
Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Ser Gly Ala Asp	
85 90 95	
cgc ctg atg acc gag cgt tgc aag gag agg gtg aac gct ttg gcc att	336
Arg Leu Met Thr Glu Arg Cys Lys Glu Arg Val Asn Ala Leu Ala Ile	
100 105 110	
gcc gtg atg aac atg tgg ccc gga gtg cgc cta cga gtg act gag ggc	384
Ala Val Met Asn Met Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly	
115 120 125	
tgg gac gag gac ggc cac cac gct cag gat tca ctc cac tac gaa ggc	432
Trp Asp Glu Asp Gly His His Ala Gln Asp Ser Leu His Tyr Glu Gly	
130 135 140	
cgt gct ttg gac atc act acg tct gac cgc gac cgc aac aag tat ggg	480
Arg Ala Leu Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys Tyr Gly	
145 150 155 160	
ttg ctg gcg cgc ctc gca gtg gaa gcc ggc ttc gac tgg gtc tac tac	528
Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr	
165 170 175	
gag tcc cgc aac cac gtc cac gtg tcg gtc aaa gct gat aac tca ctg	576
Glu Ser Arg Asn His Val His Val Ser Val Lys Ala Asp Asn Ser Leu	
180 185 190	
gcg gtc cgg gcg ggc ggc tgc ttt ccg gga aat gca act gtg cgc ctg	624
Ala Val Arg Ala Gly Gly Cys Phe Pro Gly Asn Ala Thr Val Arg Leu	
195 200 205	
tgg agc ggc gag cgg aaa ggg ctg cgg gaa ctg cac cgc gga gac tgg	672
Trp Ser Gly Glu Arg Lys Gly Leu Arg Glu Leu His Arg Gly Asp Trp	
210 215 220	
gtt ttg gcg gcc gat gcg tca ggc cgg gtg gtg ccc acg ccg gtg ctg	720
Val Leu Ala Ala Asp Ala Ser Gly Arg Val Val Pro Thr Pro Val Leu	
225 230 235 240	
ctc ttc ctg gac cgg gac ttg cag cgc cgg gct tca ttt gtg gct gtg	768
Leu Phe Leu Asp Arg Asp Leu Gln Arg Arg Ala Ser Phe Val Ala Val	
245 250 255	
gag acc gag tgg cct cca cgc aaa ctg ttg ctc acg ccc tgg cac ctg	816
Glu Thr Glu Trp Pro Pro Arg Lys Leu Leu Leu Thr Pro Trp His Leu	
260 265 270	
gtg ttt gcc gct cga ggg ccg gcg ccc gcg cca ggc gac ttt gca ccg	864
Val Phe Ala Ala Arg Gly Pro Ala Pro Ala Pro Gly Asp Phe Ala Pro	
275 280 285	
gtg ttc gcg cgc cgg cta cgc gct ggg gac tcg gtg ctg gcg ccc ggc	912
Val Phe Ala Arg Arg Leu Arg Ala Gly Asp Ser Val Leu Ala Pro Gly	
290 295 300	
ggg gat gcg ctt cgg cca gcg cgc gtg gcc cgt gtg gcg cgg gag gaa	960
Gly Asp Ala Leu Arg Pro Ala Arg Val Ala Arg Val Ala Arg Glu Glu	
305 310 315 320	

SUBSTITUTE SHEET (RULE 26)

17/44

gcc gtg ggc gtg ttc gcg ccg ctc acc gcg cac ggg acg ctg ctg gtg 1008
 Ala Val Gly Val Phe Ala Pro Leu Thr Ala His Gly Thr Leu Leu Val
 325 330 335

aac gat gtc ctg gcc tct tgc tac gcg gtt ctg gag agt cac cag tgg 1056
 Asn Asp Val Leu Ala Ser Cys Tyr Ala Val Leu Glu Ser His Gln Trp
 340 345 350

gcg cac cgc gct ttt gcc ccc ttg aga ctg ctg cac gcg cta ggg gcg 1104
 Ala His Arg Ala Phe Ala Pro Leu Arg Leu Leu His Ala Leu Gly Ala
 355 360 365

ctg ctc ccc ggc ggg gcc gtc cag ccg act ggc atg cat tgg tac tct 1152
 Leu Leu Pro Gly Gly Ala Val Gln Pro Thr Gly Met His Trp Tyr Ser
 370 375 380

cgg ctc ctc tac cgc tta gcg gag gag cta ctg ggc tg 1190
 Arg Leu Leu Tyr Arg Leu Ala Glu Glu Leu Leu Gly
 385 390 395

<210> 9
 <211> 1251
 <212> DNA
 <213> Zebrafish Thh

<220>
 <221> CDS
 <222> (1)..(1248)

<400> 9

atg gac gta agg ctg cat ctg aag caa ttt gct tta ctg tgt ttt atc 48
 Met Asp Val Arg Leu His Leu Lys Gln Phe Ala Leu Leu Cys Phe Ile
 1 5 10 15

agc ttg ctt ctg acg cct tgt gga tta gcc tgt ggt cct ggt aga ggt 96
 Ser Leu Leu Leu Thr Pro Cys Gly Leu Ala Cys Gly Pro Gly Arg Gly
 20 25 30

tat gga aaa cga aga cac cca aag aaa tta acc ccg ttg gct tac aag 144
 Tyr Gly Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys
 35 40 45

caa ttc atc ccc aac gtt gct gag aaa acg ctt gga gcc agc ggc aaa 192
 Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Lys
 50 55 60

tac gaa ggc aaa atc aca agg aat tca gag aga ttt aaa gag ctg att 240
 Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Ile
 65 70 75 80

ccg aat tat aat ccc gat atc atc ttt aag gac gag gaa aac aca aac 288
 Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Asn
 85 90 95

gct gac agg ctg atg acc aag cgc tgt aag gac aag tta aat tcg ttg 336
 Ala Asp Arg Leu Met Thr Lys Arg Cys Lys Asp Lys Leu Asn Ser Leu
 100 105 110

gcc ata tcc gtc atg aac cac tgg ccc ggc gtg aaa ctg cgc gtc act 384
 Ala Ile Ser Val Met Asn His Trp Pro Gly Val Lys Leu Arg Val Thr

SUBSTITUTE SHEET (RULE 26)

18/44

115	120	125	
gaa ggc tgg gat gag gat ggt cac cat tta gaa gaa tct ttg cac tat Glu Gly Trp Asp Glu Asp Gly His His Leu Glu Glu Ser Leu His Tyr 130 135 140			432
gag gga cgg gca gtg gac atc act acc tca gac agg gat aaa agc aag Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Lys Ser Lys 145 150 155 160			480
tat ggg atg cta tcc agg ctt gca gtg gag gca gga ttc gac tgg gtc Tyr Gly Met Leu Ser Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val 165 170 175			528
tat tat gaa tct aaa gcc cac ata cac tgc tct gtc aaa gca gaa aat Tyr Tyr Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn 180 185 190			576
tca gtg gct gct aaa tca gga gga tgt ttt cct ggg tct ggg acg gtg Ser Val Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Gly Thr Val 195 200 205			624
aca ctt ggt gat ggg acg agg aaa ccc atc aaa gat ctt aaa gtg ggc Thr Leu Gly Asp Gly Thr Arg Lys Pro Ile Lys Asp Leu Lys Val Gly 210 215 220			672
gac cgg gtt ttg gct gca gac gag aag gga aat gtc tta ata agc gac Asp Arg Val Leu Ala Ala Asp Glu Lys Gly Asn Val Leu Ile Ser Asp 225 230 235 240			720
ttt att atg ttt ata gac cac gat ccg aca acg aga agg caa ttc atc Phe Ile Met Phe Ile Asp His Asp Pro Thr Thr Arg Arg Gln Phe Ile 245 250 255			768
gtc atc gag acg tca gaa cct ttc acc aag ctc acc ctc act gcc gcg Val Ile Glu Thr Ser Glu Pro Phe Thr Lys Leu Thr Leu Thr Ala Ala 260 265 270			816
cac cta gtt ttc gtt gga aac tct tca gca gct tcg ggt ata aca gca His Leu Val Phe Val Gly Asn Ser Ser Ala Ala Ser Gly Ile Thr Ala 275 280 285			864
aca ttt gcc agc aac gtg aag cct gga gat aca gtt tta gtg tgg gaa Thr Phe Ala Ser Asn Val Lys Pro Gly Asp Thr Val Leu Val Trp Glu 290 295 300			912
gac aca tgc gag agc ctc aag agc gtt aca gtg aaa agg att tac act Asp Thr Cys Glu Ser Leu Lys Ser Val Thr Val Lys Arg Ile Tyr Thr 305 310 315 320			960
gag gag cac gag ggc tct ttt gcg cca gtc acc gcg cac gga acc ata Glu Glu His Glu Gly Ser Phe Ala Pro Val Thr Ala His Gly Thr Ile 325 330 335			1008
ata gtg gat cag gtg ttg gca tcg tgc tac gcg gtc att gag aac cac Ile Val Asp Gln Val Leu Ala Ser Cys Tyr Ala Val Ile Glu Asn His 340 345 350			1056
aaa tgg gca cat tgg gct ttt gcg ccg gtc agg ttg tgt cac aag ctg Lys Trp Ala His Trp Ala Phe Ala Pro Val Arg Leu Cys His Lys Leu			1104

SUBSTITUTE SHEET (RULE 26)

19/44

355	360	365	
atg acg tgg ctt ttt ccg gct cgt gaa tca aac gtc aat ttt cag gag			1152
Met Thr Trp Leu Phe Pro Ala Arg Glu Ser Asn Val Asn Phe Gln Glu			
370	375	380	
gat ggt atc cac tgg tac tca aat atg ctg ttt cac atc ggc tct tgg			1200
Asp Gly Ile His Trp Tyr Ser Asn Met Leu Phe His Ile Gly Ser Trp			
385	390	395	400
ctg ctg gac aga gac tct ttc cat cca ctc ggg att tta cac tta agt			1248
Leu Leu Asp Arg Asp Ser Phe His Pro Leu Gly Ile Leu His Leu Ser			
	405	410	415
tga			1251
<210> 10			
<211> 425			
<212> PRT			
<213> chicken Shh			
<400> 10			
Met Val Glu Met Leu Leu Leu Thr Arg Ile Leu Leu Val Gly Phe Ile			
1	5	10	15
Cys Ala Leu Leu Val Ser Ser Gly Leu Thr Cys Gly Pro Gly Arg Gly			
	20	25	30
Ile Gly Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys			
	35	40	45
Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg			
	50	55	60
Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr			
	65	70	75
Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly			
	85	90	95
Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu			
	100	105	110
Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr			
	115	120	125
Glu Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr			
	130	135	140
Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys			
	145	150	155
Tyr Gly Met Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val			
	165	170	175
Tyr Tyr Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn			
	180	185	190
Ser Val Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val			
	195	200	205

SUBSTITUTE SHEET (RULE 26)

20/44

His Leu Glu His Gly Gly Thr Lys Leu Val Lys Asp Leu Ser Pro Gly
 210 215 220
 Asp Arg Val Leu Ala Ala Asp Ala Asp Gly Arg Leu Leu Tyr Ser Asp
 225 230 235 240
 Phe Leu Thr Phe Leu Asp Arg Met Asp Ser Ser Arg Lys Leu Phe Tyr
 245 250 255
 Val Ile Glu Thr Arg Gln Pro Arg Ala Arg Leu Leu Leu Thr Ala Ala
 260 265 270
 His Leu Leu Phe Val Ala Pro Gln His Asn Gln Ser Glu Ala Thr Gly
 275 280 285
 Ser Thr Ser Gly Gln Ala Leu Phe Ala Ser Asn Val Lys Pro Gly Gln
 290 295 300
 Arg Val Tyr Val Leu Gly Glu Gly Gly Gln Gln Leu Leu Pro Ala Ser
 305 310 315 320
 Val His Ser Val Ser Leu Arg Glu Glu Ala Ser Gly Ala Tyr Ala Pro
 325 330 335
 Leu Thr Ala Gln Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys
 340 345 350
 Tyr Ala Val Ile Glu Glu His Ser Trp Ala His Trp Ala Phe Ala Pro
 355 360 365
 Phe Arg Leu Ala Gln Gly Leu Leu Ala Ala Leu Cys Pro Asp Gly Ala
 370 375 380
 Ile Pro Thr Ala Ala Thr Thr Thr Thr Gly Ile His Trp Tyr Ser Arg
 385 390 395 400
 Leu Leu Tyr Arg Ile Gly Ser Trp Val Leu Asp Gly Asp Ala Leu His
 405 410 415
 Pro Leu Gly Met Val Ala Pro Ala Ser
 420 425

<210> 11
 <211> 396
 <212> PRT
 <213> murine Dhh

<400> 11
 Met Ala Leu Pro Ala Ser Leu Leu Pro Leu Cys Cys Leu Ala Leu Leu
 1 5 10 15
 Ala Leu Ser Ala Gln Ser Cys Gly Pro Gly Arg Gly Pro Val Gly Arg
 20 25 30
 Arg Arg Tyr Val Arg Lys Gln Leu Val Pro Leu Leu Tyr Lys Gln Phe
 35 40 45
 Val Pro Ser Met Pro Glu Arg Thr Leu Gly Ala Ser Gly Pro Ala Glu
 50 55 60

SUBSTITUTE SHEET (RULE 26)

21/44

Gly	Arg	Val	Thr	Arg	Gly	Ser	Glu	Arg	Phe	Arg	Asp	Leu	Val	Pro	Asn	
65					70					75					80	
Tyr	Asn	Pro	Asp	Ile	Ile	Phe	Lys	Asp	Glu	Glu	Asn	Ser	Gly	Ala	Asp	
				85					90					95		
Arg	Leu	Met	Thr	Glu	Arg	Cys	Lys	Glu	Arg	Val	Asn	Ala	Leu	Ala	Ile	
			100					105					110			
Ala	Val	Met	Asn	Met	Trp	Pro	Gly	Val	Arg	Leu	Arg	Val	Thr	Glu	Gly	
		115					120					125				
Trp	Asp	Glu	Asp	Gly	His	His	Ala	Gln	Asp	Ser	Leu	His	Tyr	Glu	Gly	
	130					135					140					
Arg	Ala	Leu	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Arg	Asn	Lys	Tyr	Gly	
145					150					155					160	
Leu	Leu	Ala	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	Tyr	Tyr	
				165					170					175		
Glu	Ser	Arg	Asn	His	Ile	His	Val	Ser	Val	Lys	Ala	Asp	Asn	Ser	Leu	
			180						185				190			
Ala	Val	Arg	Ala	Gly	Gly	Cys	Phe	Pro	Gly	Asn	Ala	Thr	Val	Arg	Leu	
		195					200					205				
Arg	Ser	Gly	Glu	Arg	Lys	Gly	Leu	Arg	Glu	Leu	His	Arg	Gly	Asp	Trp	
	210					215					220					
Val	Leu	Ala	Ala	Asp	Ala	Ala	Gly	Arg	Val	Val	Pro	Thr	Pro	Val	Leu	
225					230					235					240	
Leu	Phe	Leu	Asp	Arg	Asp	Leu	Gln	Arg	Arg	Ala	Ser	Phe	Val	Ala	Val	
				245					250					255		
Glu	Thr	Glu	Arg	Pro	Pro	Arg	Lys	Leu	Leu	Leu	Thr	Pro	Trp	His	Leu	
			260					265					270			
Val	Phe	Ala	Ala	Arg	Gly	Pro	Ala	Pro	Ala	Pro	Gly	Asp	Phe	Ala	Pro	
		275					280					285				
Val	Phe	Ala	Arg	Arg	Leu	Arg	Ala	Gly	Asp	Ser	Val	Leu	Ala	Pro	Gly	
	290					295					300					
Gly	Asp	Ala	Leu	Gln	Pro	Ala	Arg	Val	Ala	Arg	Val	Ala	Arg	Glu	Glu	
305					310					315				320		
Ala	Val	Gly	Val	Phe	Ala	Pro	Leu	Thr	Ala	His	Gly	Thr	Leu	Leu	Val	
				325					330					335		
Asn	Asp	Val	Leu	Ala	Ser	Cys	Tyr	Ala	Val	Leu	Glu	Ser	His	Gln	Trp	
			340					345					350			
Ala	His	Arg	Ala	Phe	Ala	Pro	Leu	Arg	Leu	Leu	His	Ala	Leu	Gly	Ala	
		355					360					365				
Leu	Leu	Pro	Gly	Gly	Ala	Val	Gln	Pro	Thr	Gly	Met	His	Trp	Tyr	Ser	
	370					375					380					

SUBSTITUTE SHEET (RULE 26)

22/44

Arg Leu Leu Tyr Arg Leu Ala Glu Glu Leu Met Gly
385 390 395

<210> 12
<211> 411
<212> PRT
<213> murine Ihh

<400> 12
Met Ser Pro Ala Trp Leu Arg Pro Arg Leu Arg Phe Cys Leu Phe Leu
1 5 10 15
Leu Leu Leu Leu Leu Val Pro Ala Ala Arg Gly Cys Gly Pro Gly Arg
20 25 30
Val Val Gly Ser Arg Arg Arg Pro Pro Arg Lys Leu Val Pro Leu Ala
35 40 45
Tyr Lys Gln Phe Ser Pro Asn Val Pro Glu Lys Thr Leu Gly Ala Ser
50 55 60
Gly Arg Tyr Glu Gly Lys Ile Ala Arg Ser Ser Glu Arg Phe Lys Glu
65 70 75 80
Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn
85 90 95
Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Arg Leu Asn
100 105 110
Ser Leu Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg
115 120 125
Val Thr Glu Gly Arg Asp Glu Asp Gly His His Ser Glu Glu Ser Leu
130 135 140
His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg
145 150 155 160
Asn Lys Tyr Gly Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp
165 170 175
Trp Val Tyr Tyr Glu Ser Lys Ala His Val His Cys Ser Val Lys Ser
180 185 190
Glu His Ser Ala Ala Ala Lys Thr Gly Gly Cys Phe Pro Ala Gly Ala
195 200 205
Gln Val Arg Leu Glu Asn Gly Glu Arg Val Ala Leu Ser Ala Val Lys
210 215 220
Pro Gly Asp Arg Val Leu Ala Met Gly Glu Asp Gly Thr Pro Thr Phe
225 230 235 240
Ser Asp Val Leu Ile Phe Leu Asp Arg Glu Pro Asn Arg Leu Arg Ala
245 250 255
Phe Gln Val Ile Glu Thr Gln Asp Pro Pro Arg Arg Leu Ala Leu Thr
260 265 270

SUBSTITUTE SHEET (RULE 26)

23/44

Pro Ala His Leu Leu Phe Ile Ala Asp Asn His Thr Glu Pro Ala Ala
 275 280 285

His Phe Arg Ala Thr Phe Ala Ser His Val Gln Pro Gly Gln Tyr Val
 290 295 300

Leu Val Ser Gly Val Pro Gly Leu Gln Pro Ala Arg Val Ala Ala Val
 305 310 315 320

Ser Thr His Val Ala Leu Gly Ser Tyr Ala Pro Leu Thr Arg His Gly
 325 330 335

Thr Leu Val Val Glu Asp Val Val Ala Ser Cys Phe Ala Ala Val Ala
 340 345 350

Asp His His Leu Ala Gln Leu Ala Phe Trp Pro Leu Arg Leu Phe Pro
 355 360 365

Ser Leu Ala Trp Gly Ser Trp Thr Pro Ser Glu Gly Val His Ser Tyr
 370 375 380

Pro Gln Met Leu Tyr Arg Leu Gly Arg Leu Leu Leu Glu Glu Ser Thr
 385 390 395 400

Phe His Pro Leu Gly Met Ser Gly Ala Gly Ser
 405 410

<210> 13
 <211> 437
 <212> PRT
 <213> murine Shh

<400> 13
 Met Leu Leu Leu Leu Ala Arg Cys Phe Leu Val Ile Leu Ala Ser Ser
 1 5 10 15

Leu Leu Val Cys Pro Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly
 20 25 30

Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe
 35 40 45

Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu
 50 55 60

Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn
 65 70 75 80

Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp
 85 90 95

Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile
 100 105 110

Ser Val Met Asn Gln Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly
 115 120 125

Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly
 130 135 140

SUBSTITUTE SHEET (RULE 26)

24/44

Arg	Ala	Val	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Arg	Ser	Lys	Tyr	Gly
145					150					155					160
Met	Leu	Ala	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	Tyr	Tyr
				165					170					175	
Glu	Ser	Lys	Ala	His	Ile	His	Cys	Ser	Val	Lys	Ala	Glu	Asn	Ser	Val
			180					185					190		
Ala	Ala	Lys	Ser	Gly	Gly	Cys	Phe	Pro	Gly	Ser	Ala	Thr	Val	His	Leu
		195					200					205			
Glu	Gln	Gly	Gly	Thr	Lys	Leu	Val	Lys	Asp	Leu	Arg	Pro	Gly	Asp	Arg
	210					215					220				
Val	Leu	Ala	Ala	Asp	Asp	Gln	Gly	Arg	Leu	Leu	Tyr	Ser	Asp	Phe	Leu
225					230					235					240
Thr	Phe	Leu	Asp	Arg	Asp	Glu	Gly	Ala	Lys	Lys	Val	Phe	Tyr	Val	Ile
				245					250					255	
Glu	Thr	Leu	Glu	Pro	Arg	Glu	Arg	Leu	Leu	Leu	Thr	Ala	Ala	His	Leu
			260					265					270		
Leu	Phe	Val	Ala	Pro	His	Asn	Asp	Ser	Gly	Pro	Thr	Pro	Gly	Pro	Ser
		275					280					285			
Ala	Leu	Phe	Ala	Ser	Arg	Val	Arg	Pro	Gly	Gln	Arg	Val	Tyr	Val	Val
	290					295					300				
Ala	Glu	Arg	Gly	Gly	Asp	Arg	Arg	Leu	Leu	Pro	Ala	Ala	Val	His	Ser
305					310					315					320
Val	Thr	Leu	Arg	Glu	Glu	Glu	Ala	Gly	Ala	Tyr	Ala	Pro	Leu	Thr	Ala
				325					330					335	
His	Gly	Thr	Ile	Leu	Ile	Asn	Arg	Val	Leu	Ala	Ser	Cys	Tyr	Ala	Val
			340					345					350		
Ile	Glu	Glu	His	Ser	Trp	Ala	His	Arg	Ala	Phe	Ala	Pro	Phe	Arg	Leu
		355					360					365			
Ala	His	Ala	Leu	Leu	Ala	Ala	Leu	Ala	Pro	Ala	Arg	Thr	Asp	Gly	Gly
	370					375					380				
Gly	Gly	Gly	Ser	Ile	Pro	Ala	Ala	Gln	Ser	Ala	Thr	Glu	Ala	Arg	Gly
385					390					395					400
Ala	Glu	Pro	Thr	Ala	Gly	Ile	His	Trp	Tyr	Ser	Gln	Leu	Leu	Tyr	His
				405					410					415	
Ile	Gly	Thr	Trp	Leu	Leu	Asp	Ser	Glu	Thr	Met	His	Pro	Leu	Gly	Met
			420					425					430		
Ala	Val	Lys	Ser	Ser											
		435													

<210> 14
 <211> 418
 <212> PRT

SUBSTITUTE SHEET (RULE 26)

25/44

<213> zebrafish Shh

<400> 14

Met	Arg	Leu	Leu	Thr	Arg	Val	Leu	Leu	Val	Ser	Leu	Leu	Thr	Leu	Ser	1		5		10		15
Leu	Val	Val	Ser	Gly	Leu	Ala	Cys	Gly	Pro	Gly	Arg	Gly	Tyr	Gly	Arg		20		25		30	
Arg	Arg	His	Pro	Lys	Lys	Leu	Thr	Pro	Leu	Ala	Tyr	Lys	Gln	Phe	Ile		35		40		45	
Pro	Asn	Val	Ala	Glu	Lys	Thr	Leu	Gly	Ala	Ser	Gly	Arg	Tyr	Glu	Gly		50		55		60	
Lys	Ile	Thr	Arg	Asn	Ser	Glu	Arg	Phe	Lys	Glu	Leu	Thr	Pro	Asn	Tyr	65		70		75		80
Asn	Pro	Asp	Ile	Ile	Phe	Lys	Asp	Glu	Glu	Asn	Thr	Gly	Ala	Asp	Arg		85		90		95	
Leu	Met	Thr	Gln	Arg	Cys	Lys	Asp	Lys	Leu	Asn	Ser	Leu	Ala	Ile	Ser		100		105		110	
Val	Met	Asn	His	Trp	Pro	Gly	Val	Lys	Leu	Arg	Val	Thr	Glu	Gly	Trp		115		120		125	
Asp	Glu	Asp	Gly	His	His	Phe	Glu	Glu	Ser	Leu	His	Tyr	Glu	Gly	Arg		130		135		140	
Ala	Val	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Lys	Ser	Lys	Tyr	Gly	Thr	145		150		155		160
Leu	Ser	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	Tyr	Tyr	Glu		165		170		175	
Ser	Lys	Ala	His	Ile	His	Cys	Ser	Val	Lys	Ala	Glu	Asn	Ser	Val	Ala		180		185		190	
Ala	Lys	Ser	Gly	Gly	Cys	Phe	Pro	Gly	Ser	Ala	Leu	Val	Ser	Leu	Gln		195		200		205	
Asp	Gly	Gly	Gln	Lys	Ala	Val	Lys	Asp	Leu	Asn	Pro	Gly	Asp	Lys	Val	210		215		220		
Leu	Ala	Ala	Asp	Ser	Ala	Gly	Asn	Leu	Val	Phe	Ser	Asp	Phe	Ile	Met	225		230		235		240
Phe	Thr	Asp	Arg	Asp	Ser	Thr	Thr	Arg	Arg	Val	Phe	Tyr	Val	Ile	Glu		245		250		255	
Thr	Gln	Glu	Pro	Val	Glu	Lys	Ile	Thr	Leu	Thr	Ala	Ala	His	Leu	Leu		260		265		270	
Phe	Val	Leu	Asp	Asn	Ser	Thr	Glu	Asp	Leu	His	Thr	Met	Thr	Ala	Ala		275		280		285	
Tyr	Ala	Ser	Ser	Val	Arg	Ala	Gly	Gln	Lys	Val	Met	Val	Val	Asp	Asp	290		295		300		

SUBSTITUTE SHEET (RULE 26)

26/44

Ser Gly Gln Leu Lys Ser Val Ile Val Gln Arg Ile Tyr Thr Glu Glu
305 310 315 320

Gln Arg Gly Ser Phe Ala Pro Val Thr Ala His Gly Thr Ile Val Val
325 330 335

Asp Arg Ile Leu Ala Ser Cys Tyr Ala Val Ile Glu Asp Gln Gly Leu
340 345 350

Ala His Leu Ala Phe Ala Pro Ala Arg Leu Tyr Tyr Tyr Val Ser Ser
355 360 365

Phe Leu Ser Pro Lys Thr Pro Ala Val Gly Pro Met Arg Leu Tyr Asn
370 375 380

Arg Arg Gly Ser Thr Gly Thr Pro Gly Ser Cys His Gln Met Gly Thr
385 390 395 400

Trp Leu Leu Asp Ser Asn Met Leu His Pro Leu Gly Met Ser Val Asn
405 410 415

Ser Ser

<210> 15

<211> 475

<212> PRT

<213> Homo sapien Shh

<220>

<223> Xaa at position 463 is any or unknown amino acid

<400> 15

Met Leu Leu Leu Ala Arg Cys Leu Leu Leu Val Leu Val Ser Ser Leu
1 5 10 15

Leu Val Cys Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly Lys
20 25 30

Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile
35 40 45

Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly
50 55 60

Lys Ile Ser Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr
65 70 75 80

Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp Arg
85 90 95

Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile Ser
100 105 110

Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp
115 120 125

Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly Arg
130 135 140

Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly Met

SUBSTITUTE SHEET (RULE 26)

27/44

145					150						155					160
Leu	Ala	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	Tyr	Tyr	Glu	
				165					170					175		
Ser	Lys	Ala	His	Ile	His	Cys	Ser	Val	Lys	Ala	Glu	Asn	Ser	Val	Ala	
			180					185					190			
Ala	Lys	Ser	Gly	Gly	Cys	Phe	Pro	Gly	Ser	Ala	Thr	Val	His	Leu	Glu	
		195					200					205				
Gln	Gly	Gly	Thr	Lys	Leu	Val	Lys	Asp	Leu	Ser	Pro	Gly	Asp	Arg	Val	
	210					215					220					
Leu	Ala	Ala	Asp	Asp	Gln	Gly	Arg	Leu	Leu	Tyr	Ser	Asp	Phe	Leu	Thr	
225					230					235					240	
Phe	Leu	Asp	Arg	Asp	Asp	Gly	Ala	Lys	Lys	Val	Phe	Tyr	Val	Ile	Glu	
				245					250					255		
Thr	Arg	Glu	Pro	Arg	Glu	Arg	Leu	Leu	Leu	Thr	Ala	Ala	His	Leu	Leu	
			260					265					270			
Phe	Val	Ala	Pro	His	Asn	Asp	Ser	Ala	Thr	Gly	Glu	Pro	Glu	Ala	Ser	
		275					280					285				
Ser	Gly	Ser	Gly	Pro	Pro	Ser	Gly	Gly	Ala	Leu	Gly	Pro	Arg	Ala	Leu	
	290					295					300					
Phe	Ala	Ser	Arg	Val	Arg	Pro	Gly	Gln	Arg	Val	Tyr	Val	Val	Ala	Glu	
305					310					315					320	
Arg	Asp	Gly	Asp	Arg	Arg	Leu	Leu	Pro	Ala	Ala	Val	His	Ser	Val	Thr	
				325					330					335		
Leu	Ser	Glu	Glu	Ala	Ala	Gly	Ala	Tyr	Ala	Pro	Leu	Thr	Ala	Gln	Gly	
			340					345					350			
Thr	Ile	Leu	Ile	Asn	Arg	Val	Leu	Ala	Ser	Cys	Tyr	Ala	Val	Ile	Glu	
	355						360					365				
Glu	His	Ser	Trp	Ala	His	Arg	Ala	Phe	Ala	Pro	Phe	Arg	Leu	Ala	His	
	370					375					380					
Ala	Leu	Leu	Ala	Ala	Leu	Ala	Pro	Ala	Arg	Thr	Asp	Arg	Gly	Gly	Asp	
385					390					395					400	
Ser	Gly	Gly	Gly	Asp	Arg	Gly	Gly	Gly	Gly	Gly	Arg	Val	Ala	Leu	Thr	
				405				410						415		
Ala	Pro	Gly	Ala	Ala	Asp	Ala	Pro	Gly	Ala	Gly	Ala	Thr	Ala	Gly	Ile	
			420					425					430			
His	Trp	Tyr	Ser	Gln	Leu	Leu	Tyr	Gln	Ile	Gly	Thr	Trp	Leu	Leu	Asp	
	435						440					445				
Ser	Glu	Ala	Leu	His	Pro	Leu	Gly	Met	Ala	Val	Lys	Ser	Ser	Xaa	Ser	
	450					455					460					
Arg	Gly	Ala	Gly	Gly	Gly	Ala	Arg	Glu	Gly	Ala						

SUBSTITUTE SHEET (RULE 26)

28/44

465

470

475

<210> 16

<211> 411

<212> PRT

<213> Homo sapien Ihh

<400> 16

Met Ser Pro Ala Arg Leu Arg Pro Arg Leu His Phe Cys Leu Val Leu
 1 5 10 15

Leu Leu Leu Leu Val Val Pro Ala Ala Trp Gly Cys Gly Pro Gly Arg
 20 25 30

Val Val Gly Ser Arg Arg Arg Pro Pro Arg Lys Leu Val Pro Leu Ala
 35 40 45

Tyr Lys Gln Phe Ser Pro Asn Val Pro Glu Lys Thr Leu Gly Ala Ser
 50 55 60

Gly Arg Tyr Glu Gly Lys Ile Ala Arg Ser Ser Glu Arg Phe Lys Glu
 65 70 75 80

Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn
 85 90 95

Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Arg Leu Asn
 100 105 110

Ser Leu Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg
 115 120 125

Val Thr Glu Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu
 130 135 140

His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg
 145 150 155 160

Asn Lys Tyr Gly Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp
 165 170 175

Trp Val Tyr Tyr Glu Ser Lys Ala His Val His Cys Ser Val Lys Ser
 180 185 190

Glu His Ser Ala Ala Ala Lys Thr Gly Gly Cys Phe Pro Ala Gly Ala
 195 200 205

Gln Val Arg Leu Glu Ser Gly Ala Arg Val Ala Leu Ser Ala Val Arg
 210 215 220

Pro Gly Asp Arg Val Leu Ala Met Gly Glu Asp Gly Ser Pro Thr Phe
 225 230 235 240

Ser Asp Val Leu Ile Phe Leu Asp Arg Glu Pro His Arg Leu Arg Ala
 245 250 255

Phe Gln Val Ile Glu Thr Gln Asp Pro Pro Arg Arg Leu Ala Leu Thr
 260 265 270

Pro Ala His Leu Leu Phe Thr Ala Asp Asn His Thr Glu Pro Ala Ala

SUBSTITUTE SHEET (RULE 26)

29/44

275	280	285
Arg Phe Arg Ala Thr Phe Ala Ser His Val Gln Pro Gly Gln Tyr Val		
290	295	300
Leu Val Ala Gly Val Pro Gly Leu Gln Pro Ala Arg Val Ala Ala Val		
305	310	315 320
Ser Thr His Val Ala Leu Gly Ala Tyr Ala Pro Leu Thr Lys His Gly		
	325	330 335
Thr Leu Val Val Glu Asp Val Val Ala Ser Cys Phe Ala Ala Val Ala		
	340	345 350
Asp His His Leu Ala Gln Leu Ala Phe Trp Pro Leu Arg Leu Phe His		
	355	360 365
Ser Leu Ala Trp Gly Ser Trp Thr Pro Gly Glu Gly Val His Trp Tyr		
	370	375 380
Pro Gln Leu Leu Tyr Arg Leu Gly Arg Leu Leu Leu Glu Glu Gly Ser		
	385	390 395 400
Phe His Pro Leu Gly Met Ser Gly Ala Gly Ser		
	405	410

<210> 17

<211> 396

<212> PRT

<213> Homo sapien Dhh

<400> 17

Met Ala Leu Leu Thr Asn Leu Leu Pro Leu Cys Cys Leu Ala Leu Leu		
1	5	10 15
Ala Leu Pro Ala Gln Ser Cys Gly Pro Gly Arg Gly Pro Val Gly Arg		
	20	25 30
Arg Arg Tyr Ala Arg Lys Gln Leu Val Pro Leu Leu Tyr Lys Gln Phe		
	35	40 45
Val Pro Gly Val Pro Glu Arg Thr Leu Gly Ala Ser Gly Pro Ala Glu		
	50	55 60
Gly Arg Val Ala Arg Gly Ser Glu Arg Phe Arg Asp Leu Val Pro Asn		
	65	70 75 80
Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Ser Gly Ala Asp		
	85	90 95
Arg Leu Met Thr Glu Arg Cys Lys Glu Arg Val Asn Ala Leu Ala Ile		
	100	105 110
Ala Val Met Asn Met Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly		
	115	120 125
Trp Asp Glu Asp Gly His His Ala Gln Asp Ser Leu His Tyr Glu Gly		
	130	135 140
Arg Ala Leu Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys Tyr Gly		

SUBSTITUTE SHEET (RULE 26)

30/44

145 150 155 160
 Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr
 165 170 175
 Glu Ser Arg Asn His Val His Val Ser Val Lys Ala Asp Asn Ser Leu
 180 185 190
 Ala Val Arg Ala Gly Gly Cys Phe Pro Gly Asn Ala Thr Val Arg Leu
 195 200 205
 Trp Ser Gly Glu Arg Lys Gly Leu Arg Glu Leu His Arg Gly Asp Trp
 210 215 220
 Val Leu Ala Ala Asp Ala Ser Gly Arg Val Val Pro Thr Pro Val Leu
 225 230 235 240
 Leu Phe Leu Asp Arg Asp Leu Gln Arg Arg Ala Ser Phe Val Ala Val
 245 250 255
 Glu Thr Glu Trp Pro Pro Arg Lys Leu Leu Leu Thr Pro Trp His Leu
 260 265 270
 Val Phe Ala Ala Arg Gly Pro Ala Pro Ala Pro Gly Asp Phe Ala Pro
 275 280 285
 Val Phe Ala Arg Arg Leu Arg Ala Gly Asp Ser Val Leu Ala Pro Gly
 290 295 300
 Gly Asp Ala Leu Arg Pro Ala Arg Val Ala Arg Val Ala Arg Glu Glu
 305 310 315 320
 Ala Val Gly Val Phe Ala Pro Leu Thr Ala His Gly Thr Leu Leu Val
 325 330 335
 Asn Asp Val Leu Ala Ser Cys Tyr Ala Val Leu Glu Ser His Gln Trp
 340 345 350
 Ala His Arg Ala Phe Ala Pro Leu Arg Leu Leu His Ala Leu Gly Ala
 355 360 365
 Leu Leu Pro Gly Gly Ala Val Gln Pro Thr Gly Met His Trp Tyr Ser
 370 375 380
 Arg Leu Leu Tyr Arg Leu Ala Glu Glu Leu Leu Gly
 385 390 395
 <210> 18
 <211> 416
 <212> PRT
 <213> Zebrafish Thh
 <400> 18
 Met Asp Val Arg Leu His Leu Lys Gln Phe Ala Leu Leu Cys Phe Ile
 1 5 10 15
 Ser Leu Leu Leu Thr Pro Cys Gly Leu Ala Cys Gly Pro Gly Arg Gly
 20 25 30
 Tyr Gly Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys

SUBSTITUTE SHEET (RULE 26)

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35					40					45					
Gln	Phe	Ile	Pro	Asn	Val	Ala	Glu	Lys	Thr	Leu	Gly	Ala	Ser	Gly	Lys
	50					55					60				
Tyr	Glu	Gly	Lys	Ile	Thr	Arg	Asn	Ser	Glu	Arg	Phe	Lys	Glu	Leu	Ile
65					70					75					80
Pro	Asn	Tyr	Asn	Pro	Asp	Ile	Ile	Phe	Lys	Asp	Glu	Glu	Asn	Thr	Asn
				85					90					95	
Ala	Asp	Arg	Leu	Met	Thr	Lys	Arg	Cys	Lys	Asp	Lys	Leu	Asn	Ser	Leu
			100					105					110		
Ala	Ile	Ser	Val	Met	Asn	His	Trp	Pro	Gly	Val	Lys	Leu	Arg	Val	Thr
		115					120					125			
Glu	Gly	Trp	Asp	Glu	Asp	Gly	His	His	Leu	Glu	Glu	Ser	Leu	His	Tyr
130						135					140				
Glu	Gly	Arg	Ala	Val	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Lys	Ser	Lys
145					150					155					160
Tyr	Gly	Met	Leu	Ser	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val
				165					170					175	
Tyr	Tyr	Glu	Ser	Lys	Ala	His	Ile	His	Cys	Ser	Val	Lys	Ala	Glu	Asn
			180					185					190		
Ser	Val	Ala	Ala	Lys	Ser	Gly	Gly	Cys	Phe	Pro	Gly	Ser	Gly	Thr	Val
		195					200					205			
Thr	Leu	Gly	Asp	Gly	Thr	Arg	Lys	Pro	Ile	Lys	Asp	Leu	Lys	Val	Gly
	210					215					220				
Asp	Arg	Val	Leu	Ala	Ala	Asp	Glu	Lys	Gly	Asn	Val	Leu	Ile	Ser	Asp
225					230					235					240
Phe	Ile	Met	Phe	Ile	Asp	His	Asp	Pro	Thr	Thr	Arg	Arg	Gln	Phe	Ile
				245					250					255	
Val	Ile	Glu	Thr	Ser	Glu	Pro	Phe	Thr	Lys	Leu	Thr	Leu	Thr	Ala	Ala
			260					265					270		
His	Leu	Val	Phe	Val	Gly	Asn	Ser	Ser	Ala	Ala	Ser	Gly	Ile	Thr	Ala
		275				280						285			
Thr	Phe	Ala	Ser	Asn	Val	Lys	Pro	Gly	Asp	Thr	Val	Leu	Val	Trp	Glu
	290					295					300				
Asp	Thr	Cys	Glu	Ser	Leu	Lys	Ser	Val	Thr	Val	Lys	Arg	Ile	Tyr	Thr
305					310					315					320
Glu	Glu	His	Glu	Gly	Ser	Phe	Ala	Pro	Val	Thr	Ala	His	Gly	Thr	Ile
				325					330					335	
Ile	Val	Asp	Gln	Val	Leu	Ala	Ser	Cys	Tyr	Ala	Val	Ile	Glu	Asn	His
			340					345					350		
Lys	Trp	Ala	His	Trp	Ala	Phe	Ala	Pro	Val	Arg	Leu	Cys	His	Lys	Leu

SUBSTITUTE SHEET (RULE 26)

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355	360	365	
Met Thr Trp Leu Phe Pro Ala Arg Glu Ser Asn Val Asn Phe Gln Glu			
370	375	380	
Asp Gly Ile His Trp Tyr Ser Asn Met Leu Phe His Ile Gly Ser Trp			
385	390	395	400
Leu Leu Asp Arg Asp Ser Phe His Pro Leu Gly Ile Leu His Leu Ser			
	405	410	415
 <210> 19			
<211> 1416			
<212> DNA			
<213> Drosophila HH			
 <220>			
<221> CDS			
<222> (1) .. (1413)			
 <400> 19			
atg gat aac cac agc tca gtg cct tgg gcc agt gcc gcc agt gtc acc			48
Met Asp Asn His Ser Ser Val Pro Trp Ala Ser Ala Ala Ser Val Thr			
1	5	10	15
tgt ctc tcc ctg gga tgc caa atg cca cag ttc cag ttc cag ttc cag			96
Cys Leu Ser Leu Gly Cys Gln Met Pro Gln Phe Gln Phe Gln Phe Gln			
	20	25	30
ctc caa atc cgc agc gag ctc cat ctc cgc aag ccc gca aga aga acg			144
Leu Gln Ile Arg Ser Glu Leu His Leu Arg Lys Pro Ala Arg Arg Thr			
	35	40	45
caa acg atg cgc cac att gcg cat acg cag cgt tgc ctc agc agg ctg			192
Gln Thr Met Arg His Ile Ala His Thr Gln Arg Cys Leu Ser Arg Leu			
	50	55	60
acc tct ctg gtg gcc ctg ctg ctg atc gtc ttg ccg atg gtc ttt agc			240
Thr Ser Leu Val Ala Leu Leu Leu Ile Val Leu Pro Met Val Phe Ser			
65	70	75	80
ccg gct cac agc tgc ggt cct ggc cga gga ttg ggt cgt cat agg gcg			288
Pro Ala His Ser Cys Gly Pro Gly Arg Gly Leu Gly Arg His Arg Ala			
	85	90	95
cgc aac ctg tat ccg ctg gtc ctc aag cag aca att ccc aat cta tcc			336
Arg Asn Leu Tyr Pro Leu Val Leu Lys Gln Thr Ile Pro Asn Leu Ser			
	100	105	110
gag tac acg aac agc gcc tcc gga cct ctg gag ggt gtg atc cgt cgg			384
Glu Tyr Thr Asn Ser Ala Ser Gly Pro Leu Glu Gly Val Ile Arg Arg			
	115	120	125
gat tcg ccc aaa ttc aag gac ctc gtg ccc aac tac aac agg gac atc			432
Asp Ser Pro Lys Phe Lys Asp Leu Val Pro Asn Tyr Asn Arg Asp Ile			
	130	135	140
ctt ttc cgt gac gag gaa ggc acc gga gcg gat ggc ttg atg agc aag			480
Leu Phe Arg Asp Glu Glu Gly Thr Gly Ala Asp Gly Leu Met Ser Lys			

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145	150	155	160	
cgc tgc aag gag aag cta aac gtg ctg gcc tac tcg gtg atg aac gaa	528			
Arg Cys Lys Glu Lys Leu Asn Val Leu Ala Tyr Ser Val Met Asn Glu				
165	170	175		
tgg ccc ggc atc cgg ctg ctg gtc acc gag agc tgg gac gag gac tac	576			
Trp Pro Gly Ile Arg Leu Leu Val Thr Glu Ser Trp Asp Glu Asp Tyr				
180	185	190		
cat cac ggc cag gag tcg ctc cac tac gag ggc cga gcg gtg acc att	624			
His His Gly Gln Glu Ser Leu His Tyr Glu Gly Arg Ala Val Thr Ile				
195	200	205		
gcc acc tcc gat cgc gac cag tcc aaa tac ggc atg ctc gct cgc ctg	672			
Ala Thr Ser Asp Arg Asp Gln Ser Lys Tyr Gly Met Leu Ala Arg Leu				
210	215	220		
gcc gtc gag gct gga ttc gat tgg gtc tcc tac gtc agc agg cgc cac	720			
Ala Val Glu Ala Gly Phe Asp Trp Val Ser Tyr Val Ser Arg Arg His				
225	230	235	240	
atc tac tgc tcc gtc aag tca gat tcg tcg atc agt tcc cac gtg cac	768			
Ile Tyr Cys Ser Val Lys Ser Asp Ser Ser Ile Ser Ser His Val His				
245	250	255		
ggc tgc ttc acg ccg gag agc aca gcg ctg ctg gag agt gga gtc cgg	816			
Gly Cys Phe Thr Pro Glu Ser Thr Ala Leu Leu Glu Ser Gly Val Arg				
260	265	270		
aag ccg ctc ggc gag ctc tct atc gga gat cgt gtt ttg agc atg acc	864			
Lys Pro Leu Gly Glu Leu Ser Ile Gly Asp Arg Val Leu Ser Met Thr				
275	280	285		
gcc aac gga cag gcc gtc tac agc gaa gtg atc ctc ttc atg gac cgc	912			
Ala Asn Gly Gln Ala Val Tyr Ser Glu Val Ile Leu Phe Met Asp Arg				
290	295	300		
aac ctc gag cag atg caa aac ttt gtg cag ctg cac acg gac ggt gga	960			
Asn Leu Glu Gln Met Gln Asn Phe Val Gln Leu His Thr Asp Gly Gly				
305	310	315	320	
gca gtg ctc acg gtg acg ccg gct cac ctg gtt agc gtt tgg cag ccg	1008			
Ala Val Leu Thr Val Thr Pro Ala His Leu Val Ser Val Trp Gln Pro				
325	330	335		
gag agc cag aag ctc acg ttt gtg ttt gcg cat cgc atc gag gag aag	1056			
Glu Ser Gln Lys Leu Thr Phe Val Phe Ala His Arg Ile Glu Glu Lys				
340	345	350		
aac cag gtg ctc gta cgg gat gtg gag acg ggc gag ctg agg ccc cag	1104			
Asn Gln Val Leu Val Arg Asp Val Glu Thr Gly Glu Leu Arg Pro Gln				
355	360	365		
cga gtg gtc aag ttg ggc agt gtg cgc agt aag ggc gtg gtc gcg ccg	1152			
Arg Val Val Lys Leu Gly Ser Val Arg Ser Lys Gly Val Val Ala Pro				
370	375	380		
ctg acc cgc gag ggc acc att gtg gtc aac tcg gtg gcc gcc agt tgc	1200			
Leu Thr Arg Glu Gly Thr Ile Val Val Asn Ser Val Ala Ala Ser Cys				

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385		390		395		400	
tat gcg gtg atc aac agt cag tcg ctg gcc cac tgg gga ctg gct ccc	1248						
Tyr Ala Val Ile Asn Ser Gln Ser Leu Ala His Trp Gly Leu Ala Pro							
		405		410		415	
atg cgc ctg ctg tcc acg ctg gag gcg tgg ctg ccc gcc aag gag cag	1296						
Met Arg Leu Leu Ser Thr Leu Glu Ala Trp Leu Pro Ala Lys Glu Gln							
		420		425		430	
ttg cac agt tcg ccg aag gtg gtg agc tcg gcg cag cag cag aat ggc	1344						
Leu His Ser Ser Pro Lys Val Val Ser Ser Ala Gln Gln Gln Asn Gly							
		435		440		445	
atc cat tgg tat gcc aat gcg ctc tac aag gtc aag gac tac gtg ctg	1392						
Ile His Trp Tyr Ala Asn Ala Leu Tyr Lys Val Lys Asp Tyr Val Leu							
		450		455		460	
ccg cag agc tgg cgc cac gat tga	1416						
Pro Gln Ser Trp Arg His Asp							
465		470					

<210> 20
 <211> 471
 <212> PRT
 <213> Drosophila HH

<400> 20
 Met Asp Asn His Ser Ser Val Pro Trp Ala Ser Ala Ala Ser Val Thr
 1 5 10 15
 Cys Leu Ser Leu Gly Cys Gln Met Pro Gln Phe Gln Phe Gln Phe Gln
 20 25 30
 Leu Gln Ile Arg Ser Glu Leu His Leu Arg Lys Pro Ala Arg Arg Thr
 35 40 45
 Gln Thr Met Arg His Ile Ala His Thr Gln Arg Cys Leu Ser Arg Leu
 50 55 60
 Thr Ser Leu Val Ala Leu Leu Leu Ile Val Leu Pro Met Val Phe Ser
 65 70 75 80
 Pro Ala His Ser Cys Gly Pro Gly Arg Gly Leu Gly Arg His Arg Ala
 85 90 95
 Arg Asn Leu Tyr Pro Leu Val Leu Lys Gln Thr Ile Pro Asn Leu Ser
 100 105 110
 Glu Tyr Thr Asn Ser Ala Ser Gly Pro Leu Glu Gly Val Ile Arg Arg
 115 120 125
 Asp Ser Pro Lys Phe Lys Asp Leu Val Pro Asn Tyr Asn Arg Asp Ile
 130 135 140
 Leu Phe Arg Asp Glu Glu Gly Thr Gly Ala Asp Gly Leu Met Ser Lys
 145 150 155 160
 Arg Cys Lys Glu Lys Leu Asn Val Leu Ala Tyr Ser Val Met Asn Glu

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165								170				175			
Trp	Pro	Gly	Ile	Arg	Leu	Leu	Val	Thr	Glu	Ser	Trp	Asp	Glu	Asp	Tyr
			180					185					190		
His	His	Gly	Gln	Glu	Ser	Leu	His	Tyr	Glu	Gly	Arg	Ala	Val	Thr	Ile
		195					200					205			
Ala	Thr	Ser	Asp	Arg	Asp	Gln	Ser	Lys	Tyr	Gly	Met	Leu	Ala	Arg	Leu
	210					215					220				
Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	Ser	Tyr	Val	Ser	Arg	Arg	His
225					230					235					240
Ile	Tyr	Cys	Ser	Val	Lys	Ser	Asp	Ser	Ser	Ile	Ser	Ser	His	Val	His
				245					250					255	
Gly	Cys	Phe	Thr	Pro	Glu	Ser	Thr	Ala	Leu	Leu	Glu	Ser	Gly	Val	Arg
			260					265					270		
Lys	Pro	Leu	Gly	Glu	Leu	Ser	Ile	Gly	Asp	Arg	Val	Leu	Ser	Met	Thr
		275					280					285			
Ala	Asn	Gly	Gln	Ala	Val	Tyr	Ser	Glu	Val	Ile	Leu	Phe	Met	Asp	Arg
	290					295					300				
Asn	Leu	Glu	Gln	Met	Gln	Asn	Phe	Val	Gln	Leu	His	Thr	Asp	Gly	Gly
305					310					315					320
Ala	Val	Leu	Thr	Val	Thr	Pro	Ala	His	Leu	Val	Ser	Val	Trp	Gln	Pro
				325					330					335	
Glu	Ser	Gln	Lys	Leu	Thr	Phe	Val	Phe	Ala	His	Arg	Ile	Glu	Glu	Lys
			340					345					350		
Asn	Gln	Val	Leu	Val	Arg	Asp	Val	Glu	Thr	Gly	Glu	Leu	Arg	Pro	Gln
		355					360					365			
Arg	Val	Val	Lys	Leu	Gly	Ser	Val	Arg	Ser	Lys	Gly	Val	Val	Ala	Pro
	370					375					380				
Leu	Thr	Arg	Glu	Gly	Thr	Ile	Val	Val	Asn	Ser	Val	Ala	Ala	Ser	Cys
385					390				395						400
Tyr	Ala	Val	Ile	Asn	Ser	Gln	Ser	Leu	Ala	His	Trp	Gly	Leu	Ala	Pro
				405					410					415	
Met	Arg	Leu	Leu	Ser	Thr	Leu	Glu	Ala	Trp	Leu	Pro	Ala	Lys	Glu	Gln
			420					425					430		
Leu	His	Ser	Ser	Pro	Lys	Val	Val	Ser	Ser	Ala	Gln	Gln	Gln	Asn	Gly
		435					440					445			
Ile	His	Trp	Tyr	Ala	Asn	Ala	Leu	Tyr	Lys	Val	Lys	Asp	Tyr	Val	Leu
	450					455					460				
Pro	Gln	Ser	Trp	Arg	His	Asp									
465					470										

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<210> 21
<211> 221
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: degenerate polypeptide sequence

<220>
<222> 7
<223> Gly, Ala, Val, Leu, Ile, Phe, Tyr or Trp

<220>
<222> 9
<223> Arg, His or Lys

<220>
<222> 44
<223> Gly, Ala, Val, Leu, Ile, Ser or Thr

<220>
<222> 85
<223> Gly, Ala, Val, Leu, Ile, Ser or Thr

<220>
<222> 93
<223> Lys, Arg, His, Asn or Gln

<220>
<222> 98
<223> Lys, Arg or His

<220>
<222> 112
<223> Ser, Thr, Tyr, Trp or Phe

<220>
<222> 132
<223> Lys, Arg or His

<220>
<222> 137
<223> Met, Cys, Ser or Thr

<220>
<222> 139
<223> Gly, Ala, Val, Leu, Ile, Ser or Thr

<220>
<222> 181
<223> Leu, Val, Met, Thr or Ser

<220>
<222> 183
<223> His, Phe, Tyr, Ser, Thr, Met or Cys

<220>
<222> 185
<223> Gln, Asn, Glu, or Asp

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<220>

<222> 186

<223> His, Phe, Tyr, Thr, Gln, Asn, Glu or Asp

<220>

<222> 189

<223> Gln, Asn, Glu, Asp, Thr, Ser, Met or Cys

<220>

<222> 191

<223> Ala, Gly, Cys, Leu, Val or Met

<220>

<222> 196

<223> Arg, Lys, Met, Ile, Asn, Asp, Glu, Gln, Ser, Thr or Cys

<220>

<222> 200

<223> Arg, Lys, Met or Ile

<220>

<222> 206

<223> Ala, Gly, Cys, Asp, Glu, Gln, Asn, Ser, Thr or Met

<220>

<222> 207

<223> Ala, Gly, Cys, Asp, Asn, Glu or Gln

<220>

<222> 209

<223> Arg, Lys, Met, Ile, Asn, Asp, Glu or Gln

<220>

<222> 211

<223> Leu, Val, Met or Ile

<220>

<222> 212

<223> Phe, Tyr, Thr, His or Trp

<220>

<222> 216

<223> Ile, Val, Leu or Met

<220>

<222> 217

<223> Met, Cys, Ile, Leu, Val, Thr or Ser

<220>

<222> 219

<223> Leu, Val, Met, Thr or Ser

<220>

<223> each Xaa may also be any amino acid.

<400> 21

Cys	Gly	Pro	Gly	Arg	Gly	Xaa	Gly	Xaa	Arg	Arg	His	Pro	Lys	Lys	Leu
1				5					10					15	

Thr	Pro	Leu	Ala	Tyr	Lys	Gln	Phe	Ile	Pro	Asn	Val	Ala	Glu	Lys	Thr
-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

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20										25					30				
Leu	Gly	Ala	Ser	Gly	Arg	Tyr	Glu	Gly	Lys	Ile	Xaa	Arg	Asn	Ser	Glu				
		35					40					45							
Arg	Phe	Lys	Glu	Leu	Thr	Pro	Asn	Tyr	Asn	Pro	Asp	Ile	Ile	Phe	Lys				
		50				55					60								
Asp	Glu	Glu	Asn	Thr	Gly	Ala	Asp	Arg	Leu	Met	Thr	Gln	Arg	Cys	Lys				
		65			70				75					80					
Asp	Lys	Leu	Asn	Xaa	Leu	Ala	Ile	Ser	Val	Met	Asn	Xaa	Trp	Pro	Gly				
				85					90					95					
Val	Xaa	Leu	Arg	Val	Thr	Glu	Gly	Trp	Asp	Glu	Asp	Gly	His	His	Xaa				
			100					105					110						
Glu	Glu	Ser	Leu	His	Tyr	Glu	Gly	Arg	Ala	Val	Asp	Ile	Thr	Thr	Ser				
		115					120					125							
Asp	Arg	Asp	Xaa	Ser	Lys	Tyr	Gly	Xaa	Leu	Xaa	Arg	Leu	Ala	Val	Glu				
		130				135					140								
Ala	Gly	Phe	Asp	Trp	Val	Tyr	Tyr	Glu	Ser	Lys	Ala	His	Ile	His	Cys				
		145			150				155					160					
Ser	Val	Lys	Ala	Glu	Asn	Ser	Val	Ala	Ala	Lys	Ser	Gly	Gly	Cys	Phe				
			165					170						175					
Pro	Gly	Ser	Ala	Xaa	Val	Xaa	Leu	Xaa	Xaa	Gly	Gly	Xaa	Lys	Xaa	Val				
			180				185						190						
Lys	Asp	Leu	Xaa	Pro	Gly	Asp	Xaa	Val	Leu	Ala	Ala	Asp	Xaa	Xaa	Gly				
		195				200					205								
Xaa	Leu	Xaa	Xaa	Ser	Asp	Phe	Xaa	Xaa	Phe	Xaa	Asp	Arg							
		210				215					220								

<210> 22

<211> 167

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: degenerate polypeptide sequence

<220>

<222> 7

<223> Gly, Ala, Val, Leu, Ile, Pro, Phe or Tyr

<220>

<222> 8

<223> Gly, Ala, Val, Leu or Ile

<220>

<222> 9

<223> Gly, Ala, Val, Leu, Ile, Lys, His or Arg

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<220>
<222> 12
<223> Lys, Arg or His

<220>
<222> 13
<223> Phe, Trp, Tyr or an amino acid gap

<220>
<222> 14
<223> Gly, Ala, Val, Leu, Ile or an amino acid gap

<220>
<222> 17
<223> Asn, Gln, His, Arg or Lys

<220>
<222> 19
<223> Gly, Ala, Val, Leu, Ile, Ser or Thr

<220>
<222> 22
<223> Gly, Ala, Val, Leu, Ile, Ser or Thr

<220>
<222> 27
<223> Gly, Ala, Val, Leu, Ile, Ser or Thr

<220>
<222> 29
<223> Ser, Thr, Gln or Asn

<220>
<222> 30
<223> Met, Cys, Gly, Ala, Val, Leu, Ile, Ser or Thr

<220>
<222> 31
<223> Gly, Ala, Val, Leu, Ile or Pro

<220>
<222> 33
<223> Arg, His or Lys

<220>
<222> 40
<223> Gly, Ala, Val, Leu, Ile, Pro, Arg, His or Lys

<220>
<222> 41
<223> Gly, Ala, Val, Leu, Ile, Phe or Tyr

<220>
<222> 44
<223> Arg, His or Lys

<220>
<222> 45
<223> Gly, Ala, Val, Leu, Ile, Ser or Thr

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<220>
<222> 46
<223> Thr or Ser

<220>
<222> 48
<223> Gly, Ala, Val, Leu, Ile, Asn or Gln

<220>
<222> 53
<223> Arg, His or Lys

<220>
<222> 54
<223> Asp or Glu

<220>
<222> 71
<223> Ser or Thr

<220>
<222> 79
<223> Glu, Asp, Gln or Asn

<220>
<222> 83
<223> Glu or Asp

<220>
<222> 84
<223> Arg, His or Lys

<220>
<222> 85
<223> Gly, Ala, Val, Leu or Ile

<220>
<222> 87
<223> Gly, Ala, Val, Leu, Ile, Thr or Ser

<220>
<222> 95
<223> Met, Cys, Gln, Asn, Arg, Lys or His

<220>
<222> 100
<223> Arg, His or Lys

<220>
<222> 107
<223> Trp, Phe, Tyr, Arg, His or Lys

<220>
<222> 114
<223> Gly, Ala, Val, Leu, Ile, Ser, Thr, Tyr or Phe

<220>
<222> 115
<223> Gln, Asn, Asp or Glu

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<220>
 <222> 116
 <223> Asp or Glu

<220>
 <222> 125
 <223> Gly, Ala, Val, Leu, or Ile

<220>
 <222> 134
 <223> Arg, His or Lys

<220>
 <222> 135
 <223> Asn, Gln, Thr or Ser

<220>
 <222> 139
 <223> Gly, Ala, Val, Leu, Ile, Ser, Thr, Met or Cys

<220>
 <222> 141
 <223> Gly, Ala, Val, Leu, Ile, Thr or Ser

<220>
 <222> 157
 <223> Arg, His or Lys

<220>
 <222> 158
 <223> Asn, Gln, Gly, Ala, Val, Leu or Ile

<220>
 <222> 160
 <223> Gly, Ala, Val, Leu or Ile

<220>
 <222> 162
 <223> Gly, Ala, Val, Leu, Ile, Ser, Thr or Cys

<220>
 <222> 166
 <223> Gly, Ala, Val, Leu, Ile, Thr or Ser

<220>
 <222> 167
 <223> Asp or Glu

<400> 22
 Cys Gly Pro Gly Arg Gly Xaa Xaa Xaa Arg Arg Xaa Xaa Xaa Pro Lys
 1 5 10 15
 Xaa Leu Xaa Pro Leu Xaa Tyr Lys Gln Phe Xaa Pro Xaa Xaa Xaa Glu
 20 25 30
 Xaa Thr Leu Gly Ala Ser Gly Xaa Xaa Glu Gly Xaa Xaa Xaa Arg Xaa
 35 40 45
 Ser Glu Arg Phe Xaa Xaa Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile
 50 55 60

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Phe Lys Asp Glu Glu Asn Xaa Gly Ala Asp Arg Leu Met Thr Xaa Arg
 65 70 75 80
 Cys Lys Xaa Xaa Xaa Asn Xaa Leu Ala Ile Ser Val Met Asn Xaa Trp
 85 90 95
 Pro Gly Val Xaa Leu Arg Val Thr Glu Gly Xaa Asp Glu Asp Gly His
 100 105 110
 His Xaa Xaa Xaa Ser Leu His Tyr Glu Gly Arg Ala Xaa Asp Ile Thr
 115 120 125
 Thr Ser Asp Arg Asp Xaa Xaa Lys Tyr Gly Xaa Leu Xaa Arg Leu Ala
 130 135 140
 Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu Ser Xaa Xaa His Xaa
 145 150 155 160
 His Xaa Ser Val Lys Xaa Xaa
 165

<210> 23
 <211> 74
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: primer

<400> 23
 gcgcgcttcg aagcgaggca gccagcgagg gagagagcga gcgggcgagc cggagcgagg 60
 aaatcgatgc gcgc 74

<210> 24
 <211> 74
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: primer

<400> 24
 gcgcgcagat ctgggaaagc gcaagagaga gcgcacacgc acacacccgc cgcgcgact 60
 cgggatccgc gcgc 74

<210> 25
 <211> 996
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: gene
 activation construct

<400> 25

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cgaagcgagg cagccagcga gggagagagc gagcgggcca gccggagcga ggaaatcgaa 60
 ggttcgaatc cttccccac caccatcact ttcaaaagtc cgaaagaatc tgctccctgc 120
 ttgtgtgttg gaggtcgctg agtagtgccg gagtaaaatt taagctacaa caaggcaagg 180
 cttgaccgac aattgcatga agaatctgct tagggttagg cgttttgccg tgcttcgcga 240
 tgtacggggc agatatacgc gttgacattg attattgact agttattaat agtaatcaat 300
 tacgggggtca ttagttcata gcccatatat ggagttccgc gttacataac ttacggtaaa 360
 tggcccgccct ggctgaccgc ccaacgaccc ccgcccattg acgtcaataa tgacgtatgt 420
 tcccatagta acgccaatag ggactttcca ttgacgtcaa tgggtggact atttacggta 480
 aactgcccac ttggcagtac atcaagtgtg tcatatgccg agtacgcccc ctattgacgt 540
 caatgacggt aaatggcccg cctggcatta tgcccagtac atgaccttat gggactttcc 600
 tacttggcag tacatctacg tattagtcac cgctattacc atggtgatgc ggttttggca 660
 gtacatcaat gggcgtggat agcggtttga ctcacgggga tttccaagtc tccaccccat 720
 tgacgtcaat gggagtttgt tttggcacca aaatcaacgg gactttccaa aatgtcgtaa 780
 caactccgcc ccattgacgc aaatgggcgg taggcgtgta cgggtgggagg tctatataag 840
 cagagctctc tggctaacta gagaaccac tgcttactgg cttatcgaaa ttaatacgac 900
 tcactatagg gagaccaag cttggtaccg agctcggatc gatctgggaa agcgcaagag 960
 agagcgcaca cgcacacacc cgccgcgcgc actcgg 996

<210> 26
 <211> 26
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: antisense
 construct

<400> 26
 gtccctggcgc cgccgccgcc gtcgcc 26

<210> 27
 <211> 26
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: antisense
 construct

<400> 27
 ttccgatgac cggcctttcg cggtga 26

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<210> 28

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: antisense
construct

<400> 28

gtgcacggaa aggtgcaggc cacact

26

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/US99/26334 (22) International Filing Date: 8 November 1999 (08.11.99) (30) Priority Data: 09/187,387 6 November 1998 (06.11.98) US (71) Applicants (for all designated States except US): BIOGEN, INC. [US/US]; Fourteen Cambridge Center, Cambridge, MA 02142 (US). ONTOGENY, INC. [US/US]; 45 Moulton Street, Cambridge, MA 02138 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): GALDES, Alphonse [US/US]; 1 Douglas Road, Lexington, MA 02173 (US). MAHANTHAPPA, Nagesh [US/US]; 240 Norfolk Street, Cambridge, MA 02139 (US). (74) Agents: VINCENT, Matthew, P. et al.; Foley, Hoag & Eliot, LLP, One Post Office Square, Boston, MA 02109 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> (88) Date of publication of the international search report: 9 November 2000 (09.11.00)
(54) Title: METHODS AND COMPOSITIONS FOR TREATING OR PREVENTING PERIPHERAL NEUROPATHIES (57) Abstract The present application is directed to the discovery that hedgehog gene products are able to protect peripheral nerve cells under conditions which otherwise result in peripheral neuropathy. Certain aspects of the invention are directed to preparations of hedgehog polypeptides, or other molecules which regulate <i>patched</i> or <i>smoothened</i> signalling, and their uses as protective agents against both acquired and hereditary neuropathies. As used herein, "peripheral neuropathy" refers to a disorder affecting a segment of the peripheral nervous system. For instance, the method of the present invention can be used as part of a treatment program in the management of neuropathies associated with systemic disease, e.g., viral infections, diabetes, inflammation; as well as genetically acquired (hereditary) neuropathies, e.g., Charcot-Marie-Tooth disease; and neuropathies caused by a toxic agent, e.g., a chemotherapeutic agent such as vincristine.		

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/26334

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K38/17 A61K31/395 A61K31/472 A61K31/7088 A61K48/00
A61P25/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 18856 A (PRESIDENT AND FELLOWS OF HARVARD COLLEGE ET AL.) 13 July 1995 (1995-07-13) cited in the application page 3, line 18 - page 5, line 16; claims 46,50,54,57; table 1	1-15,41, 43-51
Y	page 55, line 15 - line 34 page 56, line 25 - line 30 page 57, line 11 - line 27	16-40
P, X	WO 99 10004 A (ONTOGENY, INC.) 4 March 1999 (1999-03-04)	42
Y	claims; table 1	16-40

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

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Date of the actual completion of the international search

15 June 2000

Date of mailing of the international search report

30/06/2000

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Ryckebosch, A

INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 99 04775 A (ONTOGENY, INC.) 4 February 1999 (1999-02-04)	42
Y	page 3, line 17 -page 6, line 24; claims; table 1 page 9, line 14 - line 20 page 28, line 5 - line 10 ---	16-40
P, X	WO 99 29854 A (ONTOGENY, INC.) 17 June 1999 (1999-06-17) page 44, line 28 -page 45, line 5 page 46, line 14 -page 48, line 33 ---	1-6, 44-50
A	H. ROELINK ET AL.: "FLOOR PLATE AND MOTOR NEURON INDUCTION BY DIFFERENT CONCENTRATIONS OF THE AMINO-TERMINAL CLEAVAGE PRODUCT OF SONIC HEDGEHOG AUTOPROTEOLYSIS." CELL, vol. 81, 5 May 1995 (1995-05-05), pages 445-455, XP002140279 CAMBRIDGE, MA, US cited in the application page 451, left-hand column, line 4 -page 453, left-hand column, paragraph 2 -----	1-51

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/26334

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